Delayed BCG Vaccination: Effect on the Infant Immune Response, Unrelated Pathogens and Other EPI Vaccines

Thesis

Delayed BCG vaccination: Effect on the infant immune response, unrelated pathogens and other EPI vaccines

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Dedicated to my family, especially my parents

Ebrima K Darboe and Binta Jaiteh

I am blessed and grateful to have parents as you.

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Abstract

Tuberculosis (TB) is one of the leading causes of mortality in developing countries and infants are at particular risk. Currently, the only licensed vaccine is Bacillus Calmette–Guérin (BCG), which has variable efficacy. BCG has been shown to provide heterologous protection against unrelated pathogens and enhance immunity to other Expanded Programme on Immunization (EPI) vaccines when given at birth. However, only one recently published study has shown an enhanced pro-inflammatory effect of BCG among vaccinated neonates and the immunological mechanisms of this effect in infants are not known. Further studies are required if this BCG effect is to be understood.

This thesis describes the differences in soluble and cellular responses to vaccine specific and non-specific heterologous antigens and EPI antibody levels between infants vaccinated with BCG at 6 weeks and those delayed until 18 weeks of age using flow cytometry, multiplex cytokine arrays and vaccine antibody assays.

We found low plasma cytokine levels and little cytokine production to PPD (the BCG-recall antigen) regardless of vaccination status. However, the BCG vaccinated group had lower production of IL-10 with both Toll like receptor (TLR) agonists and unrelated pathogen stimulation when compared to their unvaccinated counterparts, and this was more prominent in females than males. This contrasts with previous studies showing enhanced pro-inflammatory responses to TLR agonists and killed pathogens following BCG vaccination. The infants received BCG Denmark in these latter studies, whereas BCG Russia was used in our study, therefore strain differences in heterologous effects of BCG may be one explanation for the discrepancy.

When cellular responses were analyzed by flow cytometry low cellular responses were observed in both groups. However, we observed a significantly higher proportion of IFNγ+

CD8+ cells 1 week after vaccination compared to the unvaccinated group in response to PPD and *Candida albicans* stimulation.

Despite their different vaccine schedules, protective antibody levels to polio, tetanus and hepatitis B vaccines were obtained by all subjects; although no boosting effect of BCG was seen in contrast to previous studies when BCG was given at birth. A tuberculin skin test (TST) was performed at 18 weeks of age as a functional measure of delayed type hypersensitivity to Mtb. Due to the influence of BCG vaccination on TST induration, the vaccinated infants had significantly higher indurations than the unvaccinated infants and this was more pronounced in males.

Our findings are in contrast to a study in South Africa, but support findings from a study in Uganda, where delayed BCG vaccination significantly reduced responses to BCG compared to infants vaccinated at birth, suggesting an impact of environment (including exposure to non-tuberculous mycobacteria) in masking the immune response to BCG, differences in vaccine strains might be a major reason for differences observed in our study and the other South African studies However, two major limitations of our study are the lack of a vaccine group at birth as per the current vaccine schedule, and a post-vaccination follow up for the infants vaccinated at 18 weeks to determine differences in immunity to BCG with a further delay in vaccination.

In conclusion, BCG vaccination given at 6 weeks of age showed no difference in Th1 responses, but decreased IL-10 responses after TLR agonist and unrelated pathogen stimulation compared to unvaccinated infants. These effects, together with TST induration, were influenced by sex. Importantly, however, delaying vaccination did not have an effect on other EPI vaccine antibody levels, with all infants reaching protective levels by 18 weeks of age. This study provides further insight into BCG effects in infants in a West African setting.

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List of Abbreviations

APC	Antigen presenting cells
BFA	Brefeldin A
BCG	Bacillus Calmette Guérin
CA	*Candida albicans*
Ca^{2+}	Calcium ion
CCR7	C-C chemokine receptor 7
CFP-10	Culture Filtrate protein 10
CpG	Cytidine-phosphate Guanosine
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule –grabbing non-integrin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DTwP	Diphtheria-tetanus and whole cell pertussis
E coli	*Escherichia coli*
EDTA	Ethylene Diamine Tetra acetic acid
EPI	Expanded Program on Immunization
ESAT-6	Early secretory antigenic target 6
FCS	Fetal Calf Serum

HBV	Hepatitis B vaccine
HepA	Hepatitis A
HepB	Hepatitis B vaccine
Hib	Haemophilus influenza
HIV	Human Immunodeficiency Virus
HKLM	Heat killed *Listeria monocytogenes*
HTMV	High Titre Measles Vaccine
ICS	Intracellular Cytokine Staining
IFNγ	Interferon gamma
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
mAb	Monoclonal antibody
MBL	Mannose binding lectin
MDR	Multidrug Resistant
MHC	Major Histocompability complex
MR	Mannose receptors
M tb	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation 88
NK cells	Natural killer cells
NLR	Nod Like receptors
NLRP3	NOD-LRR-and pyrin domain-containing 3

NOD	Nucleotide-binding oligomerization domain
NTM	Non-tuberculous mycobacteria
OPV	Oral Polio Virus
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral Blood mononuclear cell
PCV-13	Pneumococcal conjugate vaccine 13
PMA	Phorbol 12-Myristate 13-acetate
PMN	Polymorphonuclear cells
PPD	Purified protein derivative
PRR	Pathogen recognition receptors
RIVM	National institute of public health, Netherlands
RPMI	Roswell Park Memorial Institute
R10	10% FCS in RPMI
SAGE	Strategic advisory group of experts
SCC	Scientific coordinating committee
SP	*Streptococcus pneumonia*
TB	Tuberculosis
T_{CM}	Central memory T cells
TCR	T cell receptors
T_{EM}	Effector memory T cells
Tfh	T follicular helper cells

Th cells	T helper cells
TIR	Toll/IL-1 receptor
TLR	Toll like receptors
TNF-α	Tumor necrosis factor α
Tregs	Regulatory T cells
TST	Tuberculin Skin Test
TT	Tetanus toxoid
WHO	World health organization
XDR	Extensively Drug resistant

1. Introduction:

1.1 Tuberculosis

1.1.1 Epidemiology of tuberculosis

Tuberculosis (TB) is a significant public health problem and a third of the world is estimated to be latently infected (WHO, 2012) with the highest burden in sub-Saharan Africa and South-East Asia(Fig 1-1). The latest WHO report estimates almost 9 million new cases of TB occur per annum with 1.4 million deaths (WHO, 2012); almost half of which are HIV associated (Fig 1-2).

Transmission occurs within air droplets (aerosols), which enter alveolar passages of exposed individuals and are ingested by alveolar macrophages. Standard pulmonary tuberculosis treatment requires 2 months intense phase therapy with rifampicin, isoniazid, ethambutol and pyrazinamide followed by 4 months of continuation phase with rifampicin and isoniazid (CDC, 2011). Fortunately most people get cured after using these drugs however the increasing prevalence of multiple drug resistant (MDR) TB and extensively drug-resistant (XDR) TB strains has exacerbated the problem (Raviglione & Smith, 2007). Multidrug resistant TB (MDR-TB) is caused by organisms that are resistant to isoniazid and rifampicin, which are the two most effective drugs against TB. Half a million cases of MDR-TB were estimated to have occurred in 2011, and 60% of these came from Brazil, China, the Russian federation, India and South Africa (WHO, 2012). More worrying is the emergence of XDR TB which is defined as TB resistant to at least four core anti-TB drugs. These generally include the two most powerful, plus resistance to any of the fluoroquinolones (e.g. ciprofloxacin) and at least one of the injectable second-line drugs (e.g. amikacin). By March of 2013, 84 countries had reported at least one case of XDR-TB (WHO, 2012).

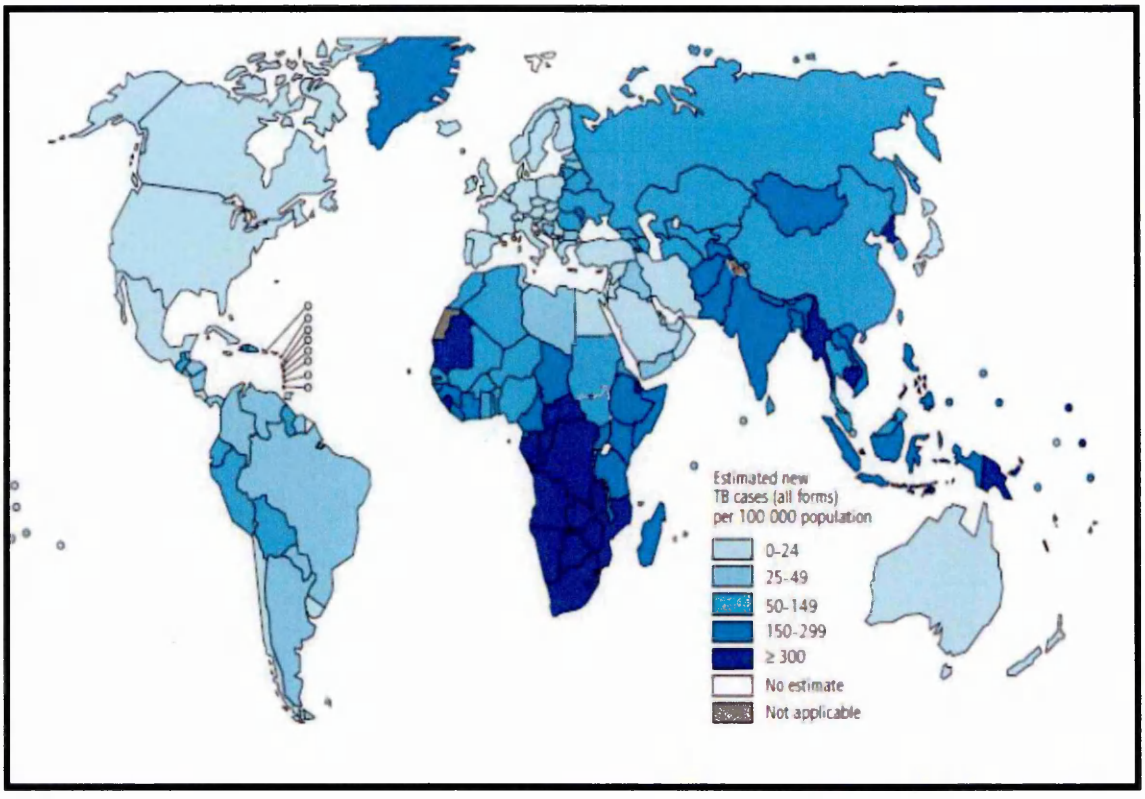


Figure 1-1: Estimated Global TB Incidence; WHO world TB report 2012). Highest disease burdens in sub-Saharan and South-east Asia

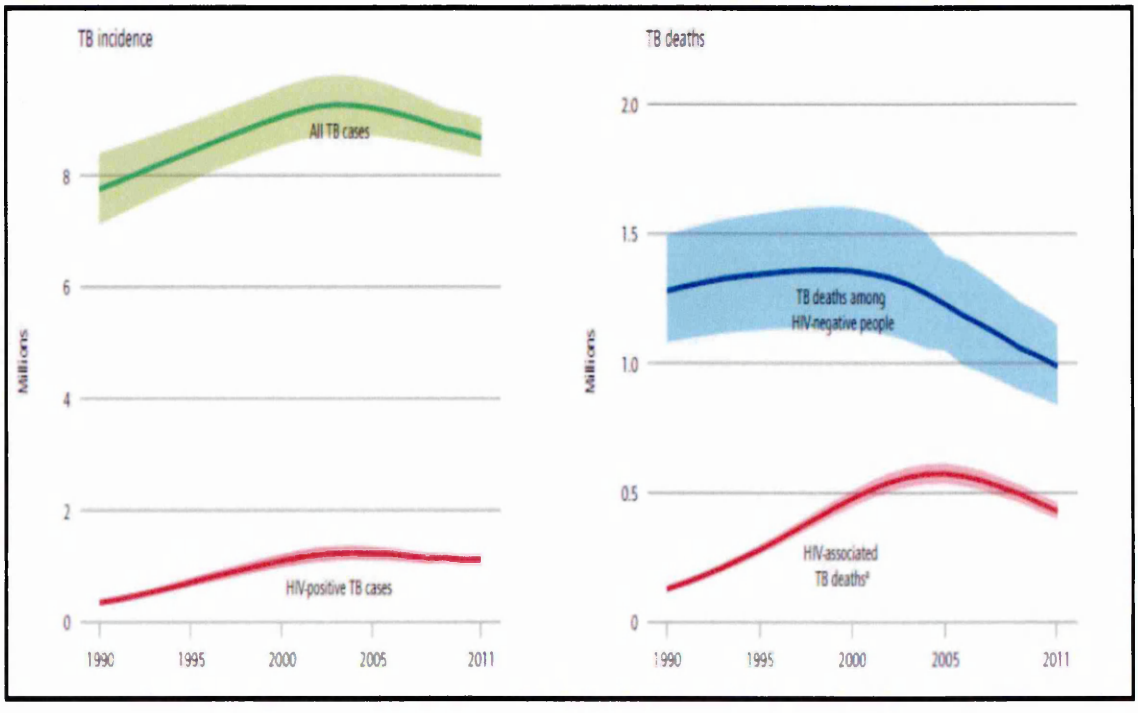


Figure 1-2: Absolute numbers of estimated global TB incidence and deaths, despite decrease in deaths there has been an increase in ; WHO world TB report 2012

1.1.2 The immune response in tuberculosis

1.1.2.1 Innate immune response

The innate immune response is the first line of defense of the immune system. Several studies suggest that innate defenses play a significant role in the immune response to tuberculosis(Ernst, 1998; Kaufmann, 2001; Abel et al., 2002).

Macrophages and c-type lectins

Macrophages are long-lived phagocytes that are detrimental to many microbial pathogens. Alveolar macrophages are the first point of contact of *Mtb* in the lungs and generally the preferred habitat of the bacteria. Although sterile eradication of the bacteria is seldom achieved, activated macrophages can control the growth of the microbe (Kaufmann, 2001). A number of surface receptors have been shown to be critical for *Mtb* detection by phagocytes, including complement receptors, mannose receptors (MR), dendritic cell-specific intercellular adhesion molecule (ICAM-3)-grabbing non-integrin (DC-SIGN), mannose binding lectin (MBL) and dectin-1 (Ernst, 1998). The initial interaction with these surface receptors influences the subsequent fate of *Mtb* within the macrophage. Autophagy influences the fate of the bacteria, with activation of macrophages leading to phagosome maturation and killing of the bacteria in macrophages (Gutierrez et al., 2004). The microbe can evade destruction by macrophages through inhibition of phagosome-lysosome fusion (Armstrong & Hart, 1971).

Toll-like receptors

The control of *Mtb* infection begins with the mycobacterial structural component such as mycolic acid and peptidoglycan being recognized (Mishra et al., 2011) via pattern recognition receptors (PRRs) on immune cells. The best known of the PRRs are the Toll Like receptors (TLRs) and Nod-Like receptors (NLRs). TLRs are a family of type 1 transmembrane receptors which are characterized by an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 receptor (TIR) domain (Medzhitov et al., 1997).

TLRs expressed by macrophages and dendritic cells (DCs) recognize a wide range of bacterial molecules including lipopeptides (TLR2), lipopolysaccharide (TLR4), bacterial CpG DNA (TLR 9) and many others. The cell wall of mycobacteria have been shown to contain several proinflammatory TLR2 ligands including lipoproteins (Brightbill et al., 1999; Means et al., 1999); thus TLR2 (acting as a heterodimer with TLR1 or TLR6) recognizes invading mycobacteria leading to the stimulation of APC effector functions (reviewed in Zuniga et al., 2012). TLR9 and possibly TLR4 are also thought to be stimulated by *Mtb* (Heldwein & Fenton, 2002). Studies in mice have demonstrated a role for both TLR2 and TLR4 in the long-term control of *Mtb* infection. Abel et al showed that immune recognition by TLR4 may be involved in protective mechanisms during chronic *Mtb* infection in mice (Abel et al., 2002). Another study from the same group showed the inability of $TLR2^{-/-}$ mice to form mycobacterial granulomas, increasing susceptibility to *Mtb* infection and reducing bacterial clearance (Drennan et al., 2004).

Nod-Like Receptors

Nod-Like receptors (NLR) are cytoplasmic proteins that contain a leucine-rich region at the C-terminus and a central nucleotide-binding oligomerization domain (NOD). There are over twenty known mammalian NLRs but only two have been shown to be involved in immunity against TB. NOD2 is a receptor for bacterial peptidoglycans; it may play a role in the host immune response to *Mtb* infection as it mediates the production of α-defensins which have bactericidal activity against mycobacteria (Kobayashi et al, 2005). NOD2 has also been shown to work together with TLR2 in mediating the immune response (Ferwerda et al., 2005). The NOD-, LRR- and pyrin domain-containing 3 (NLRP3) has also been shown to recognize one or more of the ESX1-secreted substrates (Mishra et al., 2009). *Mtb* interaction with PRRs results in the activation of downstream innate immune mediators which are involved in the phagocytosis of mycobacteria and signaling pathways related to the production of IL-12, TNF-α and IL-1β production (Court et al., 2010; Carvalho et al., 2011).

Neutrophils

Neutrophils are polymorphonuclear cells (PMNs) with abundant granules in their cytoplasm that present a large amount of bactericidal molecules. They are the most abundant leukocytes in the blood, with a lifespan ranging from a few hours to up to 5 days in the bloodstream (Borregaard, 2010; Pillay et al., 2010). Neutrophils are the first immune cells that arrive at the site of infection (Raja, 2004) and can destroy the pathogen by both oxidative (oxygen dependent) and non-oxidative (oxygen independent) mechanisms via phagocytosis of the pathogen or release of microbicidal granules (Amulic et al, 2012). Oxidative mechanisms result in the generation of reactive oxygen species, whilst non-oxidative mechanism results in degranulation - the release of preformed oxidants and proteolytic enzymes from granules. Anti-microbial compounds from granules not only kill bacteria, but may also act as chemoattractants for T-cells and immature dendritic cells, which then recruit more neutrophils to the infection site as well as initiate the adaptive immune response (Burg & Pillinger, 2001). Abundant amounts of neutrophils have been detected in the bronchoalveolar lavage fluid of pulmonary TB patients, more bacilli were found in the neutrophils than macrophages collected from sputum, bronchoalveolar lavage and cavities of these patients (Eum et al., 2010). It has been suggested by Blomgran and Ernst that *in vivo* neutrophils could be important in the induction of CD4+ T cell responses by delivering *Mtb* to dendritic cells, promoting the migration of DCs and making antigen presentation much more efficient thus favoring the T cell response (Blomgran & Ernst, 2011). A recent study using micro array data from active TB patients, latent TB infection and healthy individuals, supports a role of neutrophils in pathogenesis of TB probably due to an over-activation of IFNγ and type I IFNs, showing an important role of neutrophils in TB disease pathology(Berry et al., 2010).

Natural killer Cells (NK Cells)

Natural killer cells mediate antimycobacterial activity and are important in the defense against pathogens such as viruses, bacteria and certain parasites that use cytolytic

granules and cytokine production to mediate their effector functions (Kirwan & Burshtyn, 2007). They have the ability to bind ligands on most cell surfaces via surface binding proteins; their functional activation is determined by the interaction between stimulatory and inhibitory signals (Zuniga et al., 2012). Acting as intermediaries between the innate and adaptive immune response, they stimulate dendritic cells which in turn promote T cell responses (Kirwan & Burshtyn, 2007). NK cells were shown to be recruited to the lungs in a murine model of TB, and produced IFN-γ as well as perforin, however this was deemed not protective due to lack of infection after depletion with lytic antibodies (Junqueira-kipnis et al., 2003). NK cells in humans have been shown to produce IFN-γ after 72 hour cultures with BCG with the addition of IL-2, and *Mtb* with the addition of IL-12 (Portevin et al, 2012) indicating that priming of NK cells with cytokines is sufficient to make them respond to mycobacteria.

Dendritic Cells (DCs)

Dendritic cells are phagocytes that are involved in both the innate and adaptive immune response due to their role as professional antigen presenting cells (APCs), responsible for the initiation of adaptive immune responses. In mice, depletion of DCs resulted in impairment of the CD4+ T cell response and an inability to control the bacterial load after *M tb* infection (Tian et al., 2005). As well as their role as APC, studies in humans have shown that DCs also play a role in phagocytosis of the bacteria alongside macrophages (Henderson, Watkins, & Flynn, 1997).

1.1.2.2 Adaptive immune response to Mtb

General T cells

The adaptive immune response is the second line of defense with recognition of specific antigens expressed by pathogens. Specificity is determined by antigen specific receptors on T and B lymphocytes. Adaptive immunity is further enhanced by TLR recognition of microbes, which activate APCs such as DCs. Following stimulation, T cell antigen receptors (TCRs) on naïve T cells bind to foreign peptides and the presence of Major

Histocompatibility complex class II (MHC II) on antigen presenting cells such as DCs, signaling through the TCR and APC derived co-stimulatory molecules such as CD28 and CD80/86, naïve T cells divide to become effector T cells. Depending on the local cytokine milieu, transcription factors are activated committing them to a particular T cell subset, leading to a rise in T cell numbers at about a week after initiation of response. 1-2 weeks into the response, approximately 90% of the activated T cells die, leaving behind long-lived memory T cells (reviewed by Pepper & Jenkins, 2011). Some of these T cells become effector memory T cells (T_{EM}) capable of producing cytokines within hours of T-cell receptor stimulation, or central memory T cells (T_{CM}) which are slower to respond to antigen stimulation, but when re-stimulated secrete IL-2, proliferate extensively and acquire lymphokine effector production (Pepper & Jenkins, 2011; reviewed by Sallusto, Geginat, & Lanzavecchia, 2004). Central memory cells express CCR7 (a chemokine receptor) and CD62L, have few effector functions, but home to secondary lymphoid organs, whilst the effector memory cells lose CCR7 expression, have effector function and home to inflamed non-lymphoid tissues in humans (Sallusto et al., 1999; Geginat, Sallusto, & Lanzavecchia, 2001; Sallusto et al., 2004). Central memory cells have a higher proliferative potential than effector memory cells which gives them an edge in their ability to persist *in vivo* and efficiency in mediating protection (Lanzavecchia & Sallusto, 2005).

CD4+ T cells (helper T (Th cells)) are activated by engagement of their unique TCR with Major Histocompatibility Class II (MHC II) molecules on APCs, while CD8+ T cells (Cytotoxic T lymphocytes) are activated via MHC Class I. CD4+ T cells are specialized to activate CD8+ T cells, B cells and, importantly for TB immunity, macrophages. Helper T cells have been sub-characterized into different subsets based on their cytokine secretion pattern. Th1, Th17 and T follicular helper (Tfh) cytokines are proinflammatory and are generally induced after viral, bacterial and fungal infections, whilst Th2 cytokines are anti-inflammatory and are generally induced after parasitic and helminthic infections (Sallusto & Lanzavecchia, 2009).

Regulatory T cells (Tregs) constitute approximately 5-10% of peripheral CD4+ T cells and are important for the maintenance of peripheral tolerance and regulation of immune responses by a variety of mechanisms including the secretion of the immunosuppressive cytokines IL-10 and TGF-β.

Mtb specific T cell responses

Induction of cell-mediated immunity and granuloma formation is critical in the early control of *Mtb* infection. Different subsets of T cells have been shown to be involved in this process. Granuloma formation restricts the dissemination of the bacteria providing defense for the lungs against TB. However, if granulomas break down this can lead to aerosol transmission of TB (Rook & Hernanadez-Pando, 1996).

Mycobacteria specific CD4+ T cells are typically T helper 1 (Th1) and produce pro-inflammatory cytokines such as IFN-γ and TNF-α. Various studies have shown the indispensable role that IFN-γ plays in the immune response against TB (Cooper et al., 1993; Flynn et al., 1993). It has also been shown that patients with mutations in their IFN-γ-receptor genes are susceptible to disseminated mycobacterial infections (Newport et al, 1996). Other Th1 cytokines such as TNF-α and IL-12 have also been implicated in the immune response against TB. Treatment of patients with autoimmunity with anti-TNF-α antibodies enhances susceptibility to the development of active TB (Bruns et al., 2009) and the same has been shown in patients with a defect in their IL-12 receptor gene (DeJong et al., 1998). A Th1 response is essential for protection against TB and the induction of Th2 cytokines could antagonize this response (Lucey et al, 1996), but may also reduce immunopathology. IL-10 transgenic mice had a higher *Mtb* bacterial burden (Murray et al, 1997) and anergic human TB patients produced high amounts of IL-10 both before and after treatment suggesting immune suppression of *Mtb* (Boussiotis et al., 2000). IL-4 has been shown to play a role in disease progression since higher IL-4 expression was found in patients with pulmonary cavities (van Crevel et al., 2000).

Demissie et al found not only strong early secreted antigenic target-6 (ESAT-6, one of the immunodominant TB antigens) responses in exposed individuals who later progressed to disease, but increased IL-4 production and a decreased IFN-γ production in these individuals as they progressed (Demissie et al., 2006). Both studies point to the importance of Th1/Th2 homeostasis, with a predominant Th1 response required for protective immunity.

T helper 17 (Th17) cells are a relatively recently discovered subset producing IL-17, IL-21 and IL-22, and have also been implicated in TB control in mice and humans (Khader & Cooper, 2007; Scriba et al, 2008). Although IL-17 producing cells work in concert with IFN-γ producing cells, some studies have suggested a possible protective role of Th-17 cells in the absence of IFN-γ (Wozniak et al, 2006) .

While CD4+ T cells are the major effector cells in protection to TB, CD8 knockout mice are susceptible to *Mtb*, suggesting that CD8 T cells also play a protective role (Sousa et al., 2000). In this regard *Mtb* growth *in vitro* was shown to be inhibited by human CD1d restricted CD8+ T cells (Stenger et al, 1997). Differentiation and maturation of CD8+ T cells (Cytotoxic T Lymphocytes (CTL)) is mediated by the co-stimulatory signals produced by antigen presenting cells (APC) or by CD4+ T cells and engagement of the MHC Class I receptor. CD8 T cell clones undergo rapid proliferation in the secondary lymphoid tissues following activation leading to a rise in effector cells that migrate to the periphery to resolve infection (Bannard, Kraman, & Fearon, 2009). Once activated CTL are able to kill *Mtb*-infected targeted cells. Two mechanisms have been shown to be involved in the killing of cells; perforin mediated killing seems to be crucial for elimination of infected targeted cells (Canaday et al., 2001), while the antimicrobial peptide granulysin kills intracellular mycobacteria upon release by CD8 T cells (Stenger, et al, 1998). The second mechanism is by inducing apoptosis via the Fas-mediated pathway which controls lymphocyte homeostasis by elimination of unwanted T cells (Watson et al., 2000). Although CD4+ T cells are the primary effector cells in TB, CD8+ T cells preferentially

recognized heavily infected cells suggesting this may be essential during more severe TB infections (Lewinsohn et al., 2003).

Regulatory T cells (Tregs)

In patients with TB, Tregs have been shown to accumulate and proliferate at different sites of infection (Guyot-Revol et al, 2006; Chen et al., 2007) and in the peripheral blood (Ribeiro-Rodrigues et al., 2006). Increasing numbers of Tregs in blood has been associated with disease progression (Guyot-Revol et al., 2006). In Gambian TB cases and contacts, FOXP3 (transcription factor for Tregs) levels were higher in the blood of patients than in the blood of both infected and uninfected contacts(S Burl et al., 2007).

B cell responses

B cells have been shown to be vital for immune protection against a wide range of microbes and vaccines, however little is known about their protective role in TB infection. Studies of B cell deficiency have questioned the need for B cells in protection against TB in both mice (Johnson et al., 1997) and humans (Doffinger, Patel, & Kumararatne, 2005). However, murine studies have demonstrated protective efficacy of a mAb against mycobacterial arabinomannan and heparin-binding hemaglutinin (Teitelbaum et al., 1998; Hamasur et al., 2004).

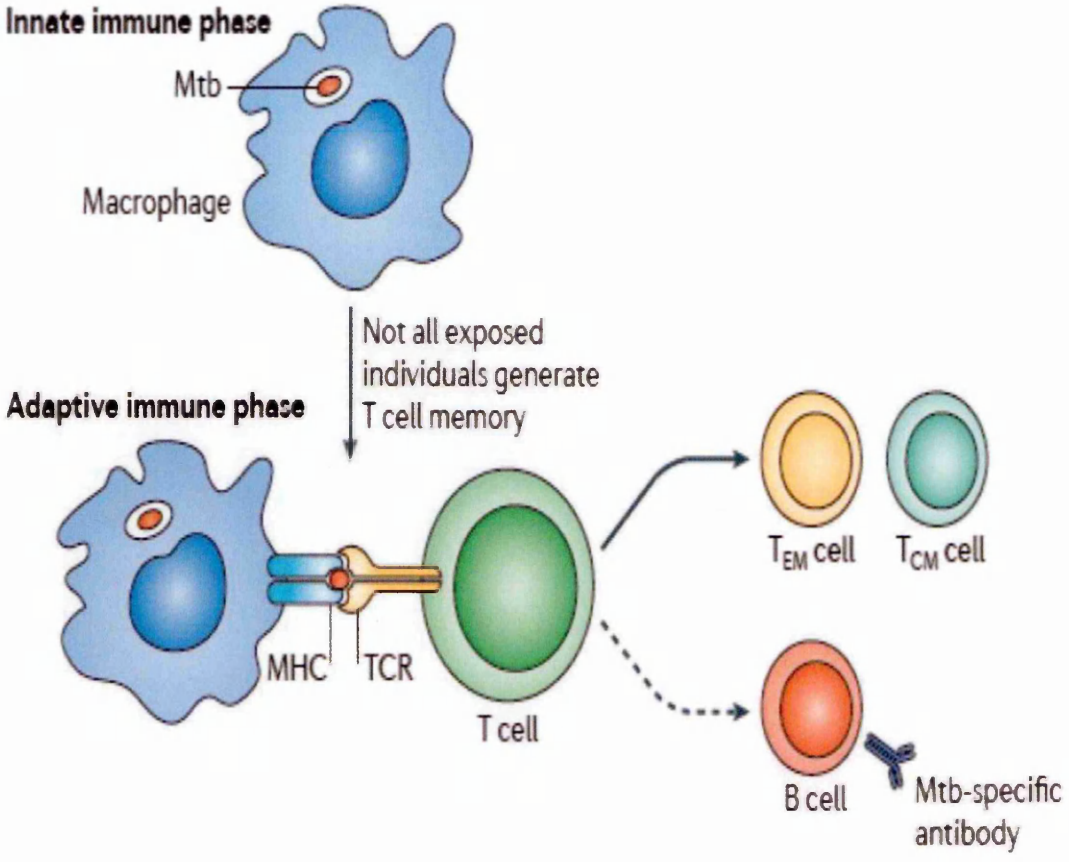


Figure 1-3: The immune response to TB. Exposure to tuberculosis results in the activation of the innate immune response. The bacterium infects the macrophages, some individuals clear the infection at this stage whilst some go on to generate T cell responses and memory (Walzl et al, NRI 2011).

1.1.2.3 Infant Immunity

While the neonatal immune system affords some protection at birth, responses are suboptimal to a range of pathogens, resulting in susceptibility of infants to severe disease (Prabhudas et al., 2011). Reduction in the innate immune response to TLRs and subsequent impairment of T cell activation, are partly responsible for reduction in acquired immunity. Interestingly, while the production of IFN-γ, IL-12p70 and IFN-α from monocytes is reduced in neonates compared to adults, neonatal mononuclear cells produce more IL-10 and IL-17 compared to adult cells (Kollmann et al., 2009), while plasma contains lower

levels of Th-1 type cytokines and higher levels of IL-6 (a Th-2 polarizing cytokine) throughout the first weeks of life in neonates (Angelone et al., 2006).

1.1.2 Vaccine-induced protection against TB

1.1.2.1 Bacillus Calmette-Guerin (BCG)

The *Mycobacterium bovis* derived vaccine, Bacillus Calmette Guerin (BCG), is the most widely used vaccine in the world today with more than 3 billion doses administered to date at an average of 120 million doses annually (Fig. 1-4). The main burden of TB is associated with adult pulmonary disease, which is also the main source of new infections. Although BCG protects against childhood miliary and pulmonary tuberculosis (Colditz et al., 1995), protection against adult pulmonary TB is widely variable (0-80%) which has been attributed to strain variation, genetic and nutritional differences between populations as well as exposure to environmental mycobacteria (Fine, 1995). WHO recommends vaccination of neonates at the birth with a single intradermal dose in high TB endemic countries, including The Gambia. However, a study comparing immunogenicity between adolescents in the UK and Malawi showed higher immunogenicity in the UK than in Malawi, thought to be due to exposure to environmental mycobacteria (Black et al., 2002).

Immaturity of the neonatal immune system has been suggested as another factor in reduced BCG efficacy (Siegrist & Kovarik, 1998). Several studies in South African children have shown higher protective responses in children who had BCG delayed to the age of 10 weeks (Hussey et al., 2002; Kagina et al., 2009). However, a study in The Gambia showed that BCG vaccination induced IFN-γ responses to adult levels regardless of whether it was given at birth or delayed to two months, but the response was higher in those vaccinated at birth (Ota et al., 2002). Another study from The Gambia showed a stronger Th1 and Th17 response in infants vaccinated at birth compared to those delayed to 4.5 months (Burl et al., 2010). This latter study attributed this difference to exposure to non-tuberculous mycobacteria, similar to the study in Malawi.

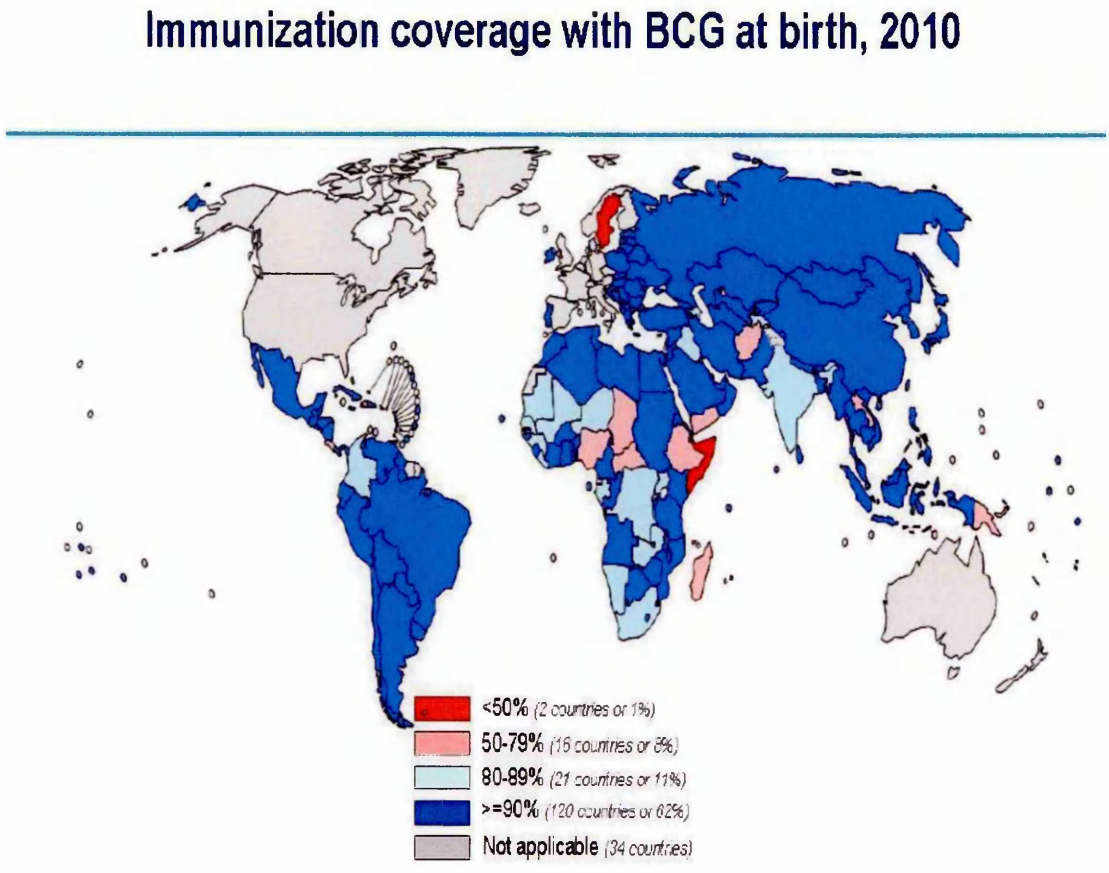


Figure 1-4: Global BCG immunization coverage; WHO 2011

1.2.1 The neonatal Immune response to BCG

The immune response to BCG is generally an adaptive response; however recent data have shown that the innate immune system is likely to play a significant role in the activation of an immune response against BCG, particularly in neonates. A South African study showed certain TLR polymorphisms were associated with an increased Th-1 type BCG induced response in whole blood ten weeks after BCG vaccination of infants (Randhawa et al., 2011). Soares et al showed that BCG is capable of inducing polyfunctional (cells producing more than 1 cytokine at the same time) CD4+ T cell responses in infants (Soares et al., 2008), including non-IFN-γ cytokines (such as IL-17 and IL-22). Similarly, in a murine model it has been shown that BCG–specific IL-17

producing T cells (Th17 cells) conferred significant protection against systemic tuberculosis in IFN-γ knockout mice (Wozniak et al, 2010). Wild type mice were able to mobilize neutrophils during the early stages of BCG infection but IL-17$^{-/-}$ mice were not, thus suggesting a role for IL-17 in the induction of neutrophils after BCG vaccination (Umemura et al., 2007). An increase in IL-17 is closely related to induction of IFN-γ responses post-BCG vaccination demonstrating its importance in BCG-induced immunity in infants and adults (Li et al., 2012).

1.3 Expanded Program on Immunization

The most successful and cost-effective public health intervention to date is immunization. National immunization programs have not only led to greatly reduced mortality and morbidity but also to the total eradication of smallpox (CDC, 2006). The World Health Organization (WHO) launched the Expanded Program on Immunization (EPI) in 1974 as part of global efforts to increase vaccination as a worldwide strategy to decrease morbidity and mortality from infections (Machingaidze, Wiysonge, & Hussey, 2013). However, despite Africa having the highest mortality rates from vaccine-preventable diseases, it still remains behind in terms of the introduction and implementation of vaccination programs (Machingaidze et al., 2013).

The Gambian EPI was introduced in 1979 following the 1978 yellow fever epidemic in the Upper River Region of The Gambia (Jobe, 2006). At present all infants are vaccinated at birth with BCG, oral polio vaccine (OPV), and Hepatitis B vaccine (HepB). At 2 months a three month intensive course is begun with the pentavalent vaccine consisting of *Haemophilus influenza* (Hib), HepB, diphtheria-tetanus and whole cell Pertusis (DTwP); alongside 3 doses of OPV, and the 13-valent pneumococcal conjugate vaccine (PCV-13) (Table 1-1). Measles and yellow fever vaccines are administered at 9 months of age with a fifth dose of OPV. At 16 months of age a booster dose of the Pentavalent vaccine is given with a final dose of OPV at 18 months (Payne, et al, 2013). Vaccine coverage has been estimated to be more than 90% by 1 year of age in Gambian infants (WHO, 2010).

Table 1-1*: The Gambia Immunization Schedule*

Age of infant	Vaccines given
Birth	BCG, Hepatitis B (HepB), Oral Polio Vaccine (OPV)
2 months	OPV, Penta (HepB, DTP, Hib), PCV-13, Rotavirus*
3 months	OPV, Penta (HepB, DTP, Hib), PCV-13, Rotavirus*
4 months	OPV, Penta (HepB, DTP, Hib), PCV-13, Rotavirus*
9 months	OPV, measles, yellow fever
16 months	DTP Booster
18 months	OPV, Measles

* introduced as part of the EPI schedule in August 2013.

1.4 Gender differences in vaccine induced immune responses

Immune responses in experimental animals have shown that females are more likely to develop a Th1 response after exposure to a pathogen, and in humans women develop higher antibody levels and stronger cell mediated immunity (Moxley et al., 2002). Adolescent Israeli females had higher measles antibody titres both before and after vaccination, with a 50% higher geometric mean post-vaccination titre when compared to males (Green et al., 1994). Following a systematic review, Cook observed that females had higher antibody levels to hepatitis A and B, rubella, measles (vaccination in adulthood) diphtheria (primary vaccination and undernourished infants) tetanus, brucella and rabies, whilst males had higher levels after pneumococcal polysaccharide (adults), diphtheria (a booster dose), measles (pre-pubertal), yellow fever, Men A and Men C (Cook, 2008). In the above mentioned review it was also observed that females had less clinical disease with influenza, Hep A and Hep B whilst males had less clinical episodes

with pneumococcal disease. In high mortality settings such as Guinea Bissau, live vaccines have been shown to provide beneficial non-specific effects (defined by Flanagan et al as "any event that could not be accounted for by induction of immunity against vaccine-targeted disease") whilst inactivated vaccines appeared to have a deleterious effect on survival (Flanagan et al., 2011). Thus mortality decreased after BCG and Measles vaccines (live vaccines) but increased after DTP (inactivated) vaccination (Aaby et al., 2012). An increase in female mortality up to 2-fold was observed in Gambia, Senegal and Guinea Bissau in infants 4 months to 5 years olds after the introduction of the High Titre Measles Vaccine (HTMV), whilst DTP in rural Guinea Bissau increased mortality in girls by up to 2.5% (Aaby et al., 2003; Aaby et al., 2004). However, this type of measles vaccine is no longer in use and a recent systematic review has not associated DTP vaccination with increased mortality (WHO, 2014).

It has been suggested that OPV could also have a gender-differential effect on mortality as missing OPV at birth was significantly associated with reduced mortality in boys and a slight tendency for increased mortality in girls (Benn et al., 2008). Several studies have observed that BCG vaccination has more beneficial effects on mortality in girls than boys (Kristensen, Aaby, & Jensen, 2000; Stensballe et al., 2005; Roth et al., 2006) .

1.5 Heterologous (or non-specific) effects of BCG

Live vaccines such as BCG and measles vaccine have been shown in some studies to have substantial survival benefits in infants leading to decreased death from infections including pneumonia and sepsis (Aaby et al., 2011). Roth et al found a correlation between the presence of a BCG scar, a positive tuberculin skin test and reduced mortality in females after controlling for socioeconomic, cultural and health-related risk factors (Roth et al., 2006).

These early observational studies have been followed by randomized trials, which confirm these effects. Thus BCG vaccination of low birth weight infants has been shown in

randomized trials to reduce subsequent mortality by 46% (Aaby et al., 2011; Biering-Sørensen et al., 2012). The immunological mechanisms behind these effects are yet to be fully elucidated but several mechanisms have been proposed. BCG vaccination of Gambian infants has been shown to boost antibody responses to other EPI vaccines including HepB, OPV and DTP (Ota et al., 2002). More recently Ritz et al found higher antibody responses to *Haemophilus influenzae* and tetanus toxoid in Australian infants vaccinated at birth than in unimmunized infants (Ritz, et al 2013). BCG vaccination has also been shown to protect against leprosy (Fine, 1999), and has a well established therapeutic effect on bladder cancer (Alexandroff, et al., 1999).

A recent study investigating the mechanism of heterologous protection against other diseases in a murine model found that the innate immune response was enhanced due to a functional reprogramming of phagocytes via a NOD-2 mediated epigenetic change in the level of histone methylation. This induced protective responses against infections other than TB such as *Candida albicans* and *E coli* LPS, and *S aureus* (Kleinnijenhuis et al., 2012). In 2012, the Strategic advisory group of experts (SAGE) on immunizations requested for WHO to review evidence on heterologous effects of vaccines. After reviewing the data SAGE concluded in July this year that the epidemiological data support beneficial effects of BCG on all-cause mortality, hence the recommendation of BCG at birth should be reinforced (WHO, 2014). Further investigation into the effect BCG has on susceptibility to other infections and the mechanisms underlying these effects are essential. Understanding these effects would help in determining strategies to optimize EPI schedules.

We hypothesize that:

- Delaying BCG until 18 weeks of age will result in lower innate and adaptive immune responses to both vaccine *and* non-vaccine related antigens compared to infants vaccinated at 6 weeks.
- The delay in BCG vaccination will also result in lower responses to TLR agonists and provide no boosting effect to EPI vaccines.
- Sustained responses to both vaccine and non-vaccine antigens will be evident in infants vaccinated at 6 weeks of age compared to those with delayed vaccination.

Project Aims

- To determine differences in the innate and adaptive immune responses at 1 week and 12 weeks) post-BCG vaccination compared to BCG-naive infants
- To establish whether BCG vaccination at 6 weeks enhances antibody responses to other EPI vaccines given at 8, 12 and 16 weeks of age.
- To establish whether BCG vaccination at 6 weeks enhances *in vitro* responses to innate stimuli and whole killed organisms at 1 and 12 weeks post-vaccination.

Chapter 2: Materials and Methods

2.1 Ethical Approval and Study Design

This study was approved by the Medical Research Council (MRC) Scientific Coordinating Committee (SCC) and the MRC/Gambia Government joint ethics committee (SCC number, 1233).

Expectant mothers presenting at Sukuta health centre were sensitized about the study, and following the birth of their baby, they were provided with specific study information and invited to join. If written informed consent was provided, babies received Hepatitis B (HepB) vaccine and the Oral Polio Vaccine (OPV, Sanofi Pasteur, France) within 48h of birth but not BCG. Exclusion criteria included low birth weight (< 2.5 kg), a baby with a congenital defect, or likelihood of moving outside the study area.

At 6 weeks of age the infants were randomized into two groups with even distribution of males and females.

- Group one had BCG vaccine at 6 weeks of age
- Group two had BCG delayed until 18 weeks of age

Apart from BCG (Serum Institute of India, India) all other vaccines were given according to The Gambian EPI schedule (Table 1-1). Every child was assessed for TB exposure at each visit, and general health information and anthropometric data were collected. 142 mothers were sensitized and 137 gave informed consent. This resulted in a total of 66 girls and 71 boys randomized to the two arms (Figure 2-1). At 8, 12 and 16 weeks all infants received Pentavalent vaccine (DTwP, Hib, HepB), OPV and PCV-13 as per the EPI schedule. Blood samples were processed within 8 hours of collection.

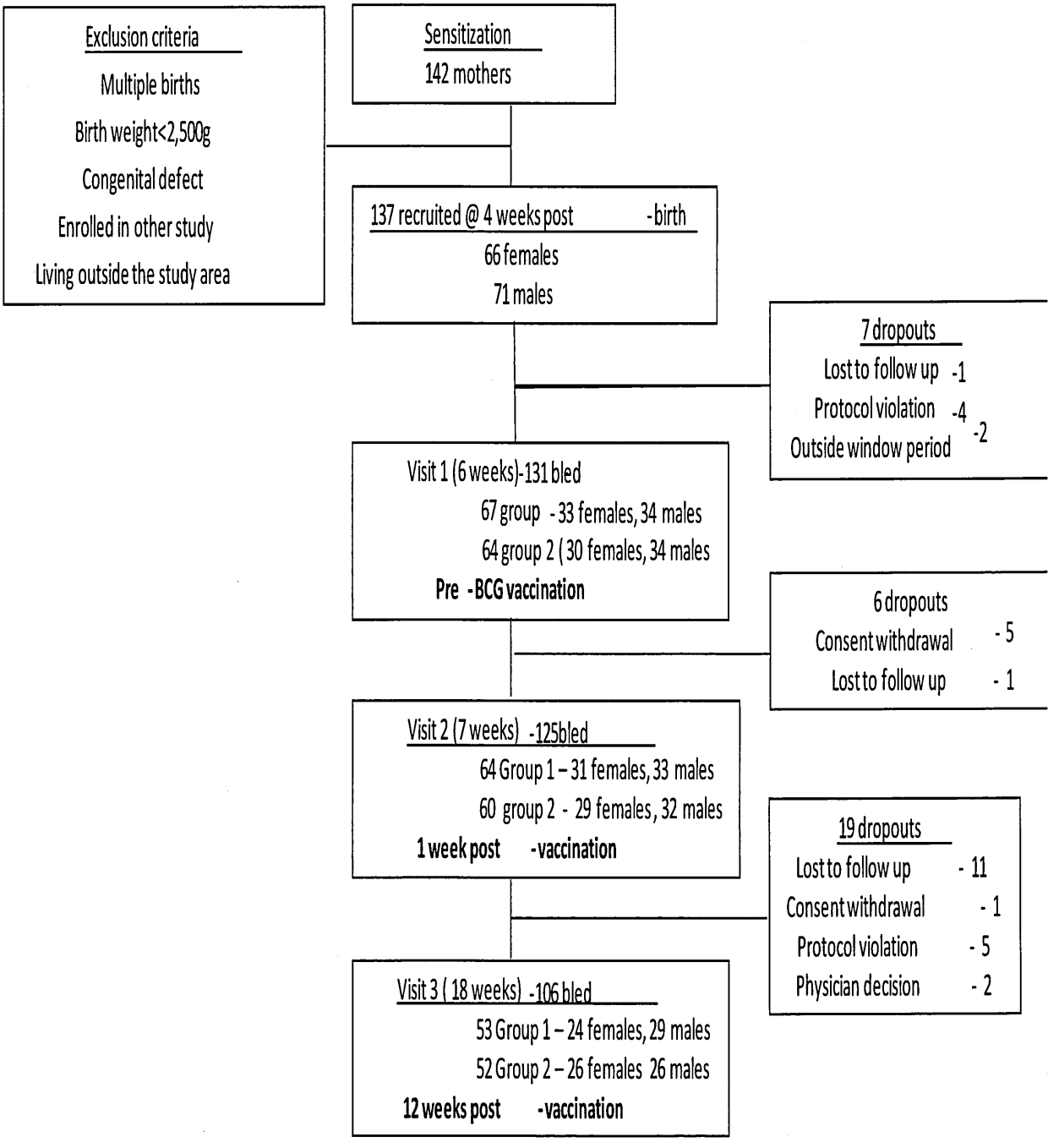


Figure 2-1: Recruitment and enrolment of participants. Mothers were sensitized, consented and recruited into the study, babies were bled at pre-vaccination visit (6 weeks), 1-week post vaccination (7 weeks), and 12-weeks post vaccination (18 weeks)

2.2 Sample collection (Figure 2-2)

4.5 mL of venous blood was collected into EDTA tubes at 6, 7 and 18 weeks by a trained fieldworker nurse using a 21 gauge needle inserted in the back of the child's hand. 0.5 mL of venous blood was collected directly into Paxgene tubes for RNA profiling to be used for determining correlations with immunological findings. These latter results will not be discussed in this thesis.

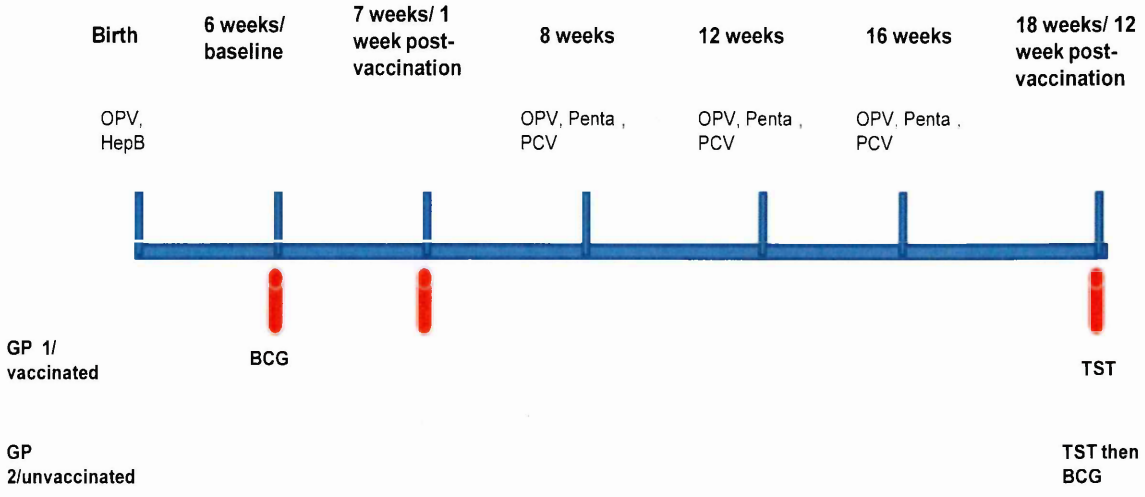


Figure 2-2: Study intervention and sampling procedures at Sukuta field site. Infants were bled at 3 times points, at time point 0 (6 weeks/baseline), 1 (7 weeks/ 1 week post-vaccination) and at 2 (18 weeks/ 12 weeks post-vaccination). All infants received the other EPI vaccines as per schedule, those in Group 1 (vaccinated group) received BCG at 0, and all infants received TST after blood was drawn at 2, infants in group 2 then received BCG after TST was read.

2.3 Tuberculin Skin Test (TST)

At 18 weeks of age a TST was performed on all participants after taking the final blood sample but before BCG vaccination of group 2. 0.1 mL of 2 tuberculin units (TU; SSI, Denmark) was injected into the infants forearm and the area marked. Induration was measured in mm both longitudinally and transversely by a trained MRC fieldworker nurse 48-72 hours later, and the average of the induration is used for analysis. A TST induration of ≥10 mm was considered suggestive of *Mtb* infection. At each visit a TB questionnaire

was completed; if anyone in the family had symptoms of TB, the family was visited and TST performed on everyone in the compound. Any individual with a positive induration >10mm was sent to a nearby TB clinic for further clinical investigation, and unvaccinated infants were immediately withdrawn, and investigated for tuberculosis infection including a chest x-ray.

2.4 Sample Processing

2.4.1 Plasma collection

Anti-coagulant treated (EDTA) whole blood was centrifuged at 1500 rpm for ten minutes. Plasma was removed and 1 mL stored at -20°C prior to analysis.

2.4.2 Vaccine antibody assays

Stored plasma samples were used to investigate EPI antibody levels using the techniques below for the different vaccines.

2.4.2.1 Polio Neutralization Antibody assay: Briefly Human Epithelial-2 (Hep-2) Cincinnati cells were thawed from Liquid Nitrogen and resuscitated in Growth Medium (10% FCS in DMEM (Dulbecco's Modified Eagle Medium (Life Technologies), with 10mM L glutamine and 10mM Pen/Strep). Freezing medium was washed off and cells counted 7mLs of the Growth medium if viability was greater than 90% in a $25cm^3$ cell culture flask. Cells were incubated for 2-3 days (until confluency was observed) at 37°C 5% CO_2, after which the cells were split using 2.5% EDTA Trypsin (Sigma, UK). During trypsinisation culture medium was poured off, and monolayer rinsed with 5 mLs 0.02% EDTA in PBS, and flaked off culture flask with 1 mL of 2.5% of EDTA Trypsin (Sigma Aldrich) after which a viability count is done. Cells were transferred into a $75cm^3$ flask and grown to confluency. On the day of the experiment, samples were diluted 1:4 with PBS and decomplemented at 56°C for 30 minutes. 50µL of maintenance medium (2% FCS in DMEM) and the same volume of diluted serum added, the first dilution being 1:8, a double dilution was done until the dilution of 1:1024. 50µL of prepared virus (virus diluted in

maintenance medium) (Polio 1 diluted to 1:1000000 from 1:100 and Polio 3 was diluted to 1:150000 from 1:100) added to all wells except the negative control and incubated for an hour at 37°C 5% CO_2. Hep-2 cells were split and diluted to a final concentration of 2 X 10^5 cells/mL in maintenance medium and 100µL of Hep-2 cells were added. Cytopathic effect (lack of a visible monolayer) of the virus on Hep-2 cells was read 5 days after incubation.

2.4.2.2 Hepatitis Antibody ELISA

A commercial kit was obtained from Diasorin (Italy) and samples were processed according to manufacturer's instructions. Briefly, plasma samples together with a positive and negative control (in kit) were aliquoted on to plates coated with Hepatitis B surface antigen (HBsAg), incubated for 1 hour at 37°C and washed using an automated plate washer (ASYS Atlantis, Biochrom, UK). A tracer solution was added and samples were incubated and washed as described above. Chromogen/substrate solution was added on to all wells, incubated for 30 minutes at room temperature in the dark after which a stop solution (in kit supply) was added and absorbance was read within 15 minutes with a spectrophotometer and fluorescence intensity read at 450 nm.

2.4.2.3 DTP Multiplex Immunoassay (MIA):

Measurement of antibody levels to diphtheria, tetanus and pertusis was done via a multiplex immunoassay developed in the serology lab after technology transfer from the national institute of public health and the environment (RIVM), Netherlands(van Gageldonk et al., 2008). Briefly, filter plates were pre-wet with PBS and standards were diluted from 1:200 to 1: 204, 800 for the pertussis antigen and 1:50 to 1:819,200 for the diphtheria and tetanus toxoids whilst samples were diluted into 1:200 and 1:4,000 dilutions. Beads conjugates with the antigens above were added on to the pre-wetted plates before 25µL of samples were added. Samples were incubated for 30 minutes on a shaker at room temperature, filter washed three times. Detection antibody is added, incubated and washed as above. R-Phycoerythrin (PE) conjugated to Goat anti-human is added on to samples, incubated and washed as above, PBS is added and samples are

shaken for 5 minutes at room temperature. Samples are read with the Bio-Plex 200 system within 15 minutes and analyzed using the Bio-Plex manager 4.0 software (Bio-Rad, Belgium).

2.4.3 Peripheral Blood Mononuclear Cell (PBMC) Separation

PBMCs were collected using the density gradient centrifugation method. Whole blood diluted 1:1 with Roswell Park Memorial institute (RPMI)(Sigma, UK) was layered on Lymphoprep (Fresenius Kabi Norge AS, Norway) and spun at 1500 rpm for 30 minutes without the brake. The PBMC layer was harvested washed in RPMI twice and cells counted. For every 10 million cells 500µL of Fetal Calf Serum (FCS)(Gibco, Life technologies) was added, left on ice for 30 minutes and another 500µL of 20% Dimethyl SulphOxide (DMSO) (Sigma Aldrich, UK) was added. The cells were transferred into a Mr. Frosty container, stored at -70°C and transferred to liquid nitrogen within 24 hours.

2.4.4 Thawing of PBMCs

PBMCs were collected from Liquid Nitrogen and thawed rapidly at 37°C in a water bath. Cells were resuspended in warm 10% FCS in RPMI (R10), and washed twice. Cells were counted, resuspended in R10 and 25 units/mL of Benzonuclease (Sigma, UK) and rested at 37°C 5% CO_2 for 6 hours with a loose lid.

2.4.5: Multi-cytokine assays

2.4.5.1 Whole blood stimulation

100µl/test of whole blood was added to 1 well of a 96-well plate and incubated at 37°C/5% CO_2 for 16 hours with PPD (10µg/mL; SSI, Denmark), tetanus toxoid (10µg/mL), LPS (TLR4 agonist; 1µg/mL; Invivogen), heat killed *Listeria monocytogenes* (TLR2 agonist; 10^9/mL; Invivogen) and CLO-75 (TLR7/8 agonist; 10µg/mL; Invivogen). After overnight incubation, 100µl of RPMI was added to each well, mixed and plate spun at 1500rpm, for 10 minutes. Supernatants were carefully harvested and stored at -20°C until required.

2.4.5.2 PBMC stimulation

1.0 x 10^6 cells were aliquoted and 0.1 x 10^6 were stimulated and incubated for 16 hours at 37°C 5% CO_2. In addition to the antigens used for the whole blood stimulation *Escherichia coli (10^6cells/mL), Streptococcus pneumoniae (10^5 cells/mL)* and *Candida albicans (10^5 cells/mL)* were used to assess response to other organisms. The antigens were obtained from Prof Mihai Netea (Radboud University Nijmegen medical). Supernatants were collected after the addition of 100µL of RPMI and centrifugation at 1500rpm for 10 minutes.

2.4.5.3 Multiplex cytokine assay

Cytokines were assessed using Bioplex Pro custom made kits (Bio-Rad, Belgium). Supernatants were spun at 1500rpm for 5 minutes to pellet any precipitation in the samples. Cytokines included Th1 (IFN-γ, TNF-α and IL-2), Th2 (IL4, IL10); Th17 (IL17); and IL-12p70 as an innate factor. IL10 can also be produced by Tregs and Th1 cells and TNF-α by innate cells (including macrophages). The assays were carried out according to the manufacturer's instructions but with a 1:2 dilution of reagents. Briefly, coupled magnetic beads were diluted 1:2 with assay buffer, and added on 96-well filter plates after plates were pre-wet. Plates were washed using a vacuum manifold station. 50µL of samples and standards were added. Lyophilized standards were diluted with culture medium, left on ice for 30 minutes and to get the first standard (S1), 128 µl of standards were diluted with 72 µL of medium. A 1:4 serial dilution followed from S1 to S8 (the standard with the lowest concentration). Plates were incubated for 30 minutes on a shaker. Detection antibodies used were diluted 1:2 and 25µL added followed by incubation at room temperature on a shaker. Streptavidin PE was added as per manufacturer's instructions, incubated for 10 mins and assay buffer added. The cytokine responses were read using the Bioplex 200 system, Luminex x-map technology and bio-plex pro software version 4.0 (Bio-Rad, Belgium). All values less than the lowest value of the standard within the standard curve were given the value of the lowest standard and all

values higher than the highest standard were given the value of the highest standard. The lowest and higher values for each analyte are as follows: cytokines (S8 (lowest)-S1 (highest)); IL-2 (0.88-14426), IL-4 (0.35-5693), IL-10 (1.45-23718), IL-12p70 (2.65-43449), IL-17 (2.33-38209), IFNγ (2.05-33560), TNFα (6.69-109604). Background (Unstimulated) results were subtracted from antigen-stimulated results to obtain specific levels of cytokines.

2.4.6: Intracellular cytokine staining

2.4.6.1 Whole Blood

350µl of whole blood was used for each test. Antigens used were purified protein derivative of *M. tuberculosis* (PPD; 10µg/mL, SSI) and Esat-6/CFP-10 (EC; 2.5µg/mL, Proimmune) as vaccine specific antigens. Controls included PMA/Ionomycin (0.1µg/1µg/mL, Sigma, UK) as a positive control and unstimulated (media alone) as a negative control. Samples were incubated for two hours prior to addition of Brefeldin A (BFA; 10µg/mL final concentration) and incubated overnight at 37°C/5% CO_2. 0.5mMol of EDTA was added and incubated for 15 minutes before red cells were lysed with RBC lysing buffer (BD, USA). PBS was later added to wash off the red cells and spun at 1500 rpm for 10 minutes. Supernatant was decanted before the addition of freezing medium (10% Dimethyl Sulphoxide (DMSO) in PBS) and cells were stored at -70°C for subsequent flow cytometry.

2.4.6.2 PBMC cultures

PBMC were resuspended to 0.5 x10^6 cells per mL per antigen; where cell numbers were too low for all stimulations priority was given to vaccine-specific responses. The same stimulants were used as for whole blood stimulations (described above) with the addition of Tetanus toxoid, *E. coli*, *S. pneumoniae* and *C. albicans.* Cells were incubated for 2 hours at 37°C, 5% CO2 after which BFA (10µg/mL) was added and cells were incubated for a further 16 hours. The next day, cells were centrifuged, supernatant was removed, and the cells stained with a surface marker cocktail consisting of CD3-PerCP cy5.5, CD3

APC efluor 750 and CD8 APC efluor 780. Cells were incubated for 30 minutes at 4°C and washed with 1mL of PBS/1% FCS/0.2% Sodium Azide (FACS buffer). Supernatant was removed and cells washed in fix/perm solution (ebioscience, UK). Permeabilisation was then performed using perm/wash solution (ebioscience, UK), followed by addition of the cytokine panel: IL-2 FITC, IL-17 PE, TNF-α PE-Cy7, IL-10 efluor-450 and IFN-γ APC. Prior to staining a Live-Dead Fixable yellow (Invitrogen, USA) stain was used in order to gate out dead cells from the analysis. Cells were acquired on a CyanADP (Beckman Coulter, USA) flow cytometer using Summit v4 software and analyzed using FlowJo v10.0.2 (Treestar, USA). At least 100,000 lymphocytes were acquired.

2.4.7 Statistical analysis

A Non-parametric Mann Whitney U test was used to assess for differences between two groups and Kruskal-Wallis test used to assess for differences between three groups. A p-value of <0.05 was considered significant. False discovery rate of 5% was used to correct for multiple testing and a q-value of <0.035 was considered significant.

Flow cytometry data was exported to Pestle (Mario Roederer, Vaccine Research Center, National Institute of Allergy and Infectious Diseases (National Institutes of Health) and Spice v5 (this software is provided free of charge by the National Institute of Allergy and Infectious Disease) for polyfunctional cytokine analysis.

Chapter 3: Optimization experiments: a comparison of cryopreserved PBMC versus EDTA-whole blood cultures

3.1 Introduction

According to the WHO, whole blood is "a venous, arterial or capillary blood sample in which the concentrations and properties of cellular and extra-cellular constituents remain relatively unaltered when compared with their in-vivo state" whilst anticoagulants are defined as "additives that inhibit blood and/or plasma from clotting ensuring that the constituent to be measured non-significantly changes prior to the analytical process" (WHO, 2002). Several types of anticoagulants are available using different mechanisms, Ethylene Diamine Tetra Acetate (EDTA) and citrate work by binding calcium ions, and the heparinates work by inhibiting thrombin activity. Stimulated whole blood collected in EDTA tubes produce very low cytokine responses compared to stimulated whole blood collected in heparin tubes using porcine blood cells (Duvigneau et al., 2007)

Calcium ions (Ca^{2+}) function as a universal second messenger in virtually all eukaryotic cells including immune cells (Feske, 2007) and signals have been described in all cells of the immune system, contributing to activation, effector functions, gene expression and differentiation. Calcium ions are essential for T cell activation following cross-linking of the T cell receptor (TCR) (Premack & Gardner, 1992). Ca^{2+} influx must be maintained for at least an hour to drive cell activation events that lead to IL-2 expression after which T cell activation becomes antigen independent (Lewis, 2001). T cells deficient for Ca^{2+} influx have impaired IL-2 production and T-cell proliferation in vitro and defective T-Cell mediated immune responses in vivo due to the lack of TCR-mediated Ca^{2+} signals (Feske, 2007).

Cryopreservation of Peripheral Blood mononuclear Cells (PBMCs) allows batch analysis of donors at different time-points, thus reducing inter-assay variability. However,

cryopreservation and thawing has been shown to differentially affect immune subsets. Proportions of CD4+ and CD8+ T cells, CD14+ monocytes, CD16+ NK Cells and CD20+ B cells do not change considerably (Allsopp & Nicholls, 1998), but naïve T cells are highly susceptible to the freeze/thawing process (Kvarnström et al , 2004) and functionality of T and NK cells is reduced (Fujiwara S, et al., 1986). Thus, analysis in fresh cells versus cryopreserved cells, and the type of anti-coagulant used will affect the downstream immune profiles generated and is an important consideration in any vaccine study.

Hypothesis:

- Blood collected in heparin tubes will have significantly greater cytokine responses following antigenic stimulation than blood collected in EDTA tubes.

- Cryopreserved PBMC from our study subjects will have better soluble cytokine levels (detected by Bioplex cytokine arrays) following antigen stimulation than supernatants stored from study subjects following stimulation of EDTA blood.

3.2 Aims

- To compare cytokine production after antigenic stimulation of whole blood collected in EDTA versus heparin to determine if there is a difference in activation with the different anti-coagulants.
- To determine if cryopreserved cells could be used in place of whole blood for soluble cytokine identification following antigen-stimulation compared to fresh PBMC, stored supernatants from EDTA blood (study subjects) and fresh whole blood.

3.3 Methods

3.3.1 Whole blood flow Cytometry

Venous blood from two adults was collected into EDTA and Heparin tubes. 200uL blood was stimulated with either PMA/Ionomycin (positive control; Sigma, UK) to a final

concentration of 0.1/1µg/mL or media alone (negative control) overnight at 37°C, 5% CO2. After the first two hours, Brefeldin A (BFA) (Sigma, UK) was added to a final concentration of 10ug/ mL to limit cytokine release from the Golgi complex. For flow cytometry analysis, cells were stained with previously titrated CD3 Pycoerythrin-Cy7, CD4 PerCP and CD8 Pacific Blue (all from Becton-Dickinson, USA) for 30 minutes at room temperature (RT) in the dark. Red cells were lysed using 2mL of 1X FACS lysing buffer (Becton Dickinson, USA) for 10 minutes at RT in the dark. Following centrifugation at 1500rpm for 5 minutes, supernatant was removed and 1X Perm 2 Buffer (Becton Dickinson, USA) was added in order to permeabilise the cells. Tubes were incubated for 20 mins at RT, centrifuged at 1800rpm for 5 minutes and supernatant removed. Cytokine antibodies were then added: IL-2 PE, TNF-α FITC and IFN-γ APC (all from e bioscience, UK) and incubated for 30 minutes at RT. Following washing, cells were resuspended in FACS buffer and acquired using a 9-colour CyanADP flow cytometer (Beckman-Coulter, USA) using Summit v.4.0 software followed by analysis with FlowJo v.7.6.3 (Treestar, USA).

3.3.2 Cell culture conditions for the Luminex assay

Venous blood was collected from five adults into EDTA and Heparin vacutainers. 100 µl of whole blood was set up for three culture conditions: PMA/Ionomycin, PPD (10µg/mL; SSI) and media alone (negative control). The remaining whole blood was layered on lymphoprep and PBMCs collected. After cell counting, 0.1 $x10^6$ PBMC per test were stimulated with the same culture conditions as above. Five PBMC samples cryopreserved previously from study subjects were thawed, counted and stimulated as above. Following overnight incubation at 37°C, 5% CO2, 100µL of RPMI was added, supernatants harvested and stored at -20°C. Corresponding supernatants previously stored from EDTA stimulated cultures of study subjects were retrieved for comparison to fresh EDTA, fresh heparin, fresh PBMC and cryopreserved PBMC to determine the optimal stimulation conditions.

3.3.3 Luminex cytokine assay

Samples were analysed using a customized Bioplex Pro cytokine kit (Bio-Rad, Belgium) as per manufacturer's instructions (See Chapter 2) for IL-1β, IL-4, IL-10, IFN-γ, TNF-α and IL-12p70. Plates were read using the Bioplex 200 system, Luminex x-map technology and bio-plex pro software version 4.0 (Bio-Rad, Belgium).

3.3.4 Statistical analysis

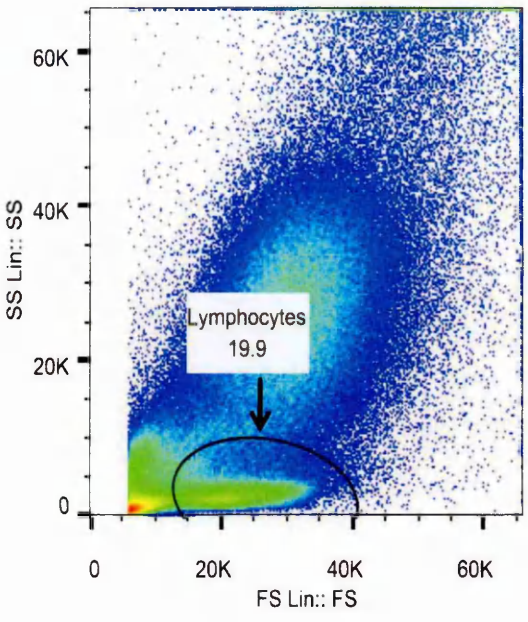
No statistical analysis was done for the flow cytometry data as data were generated from a small number of subjects for optimization only. A non-parametric Mann-Whitney U test was used to analyze for differences within groups for the Luminex assay. A p-value of <0.05 was considered significant.

3.4 Results

3.4.1 Whole blood collected in heparin tubes had a higher frequency of lymphocytes and cytokines than whole blood collected in EDTA tubes.

Differences were evident in the PMA/Ionomycin stimulated samples when the lymphocyte population was analysed (Fig 3-1), these differences might be due to the effect of EDTA interaction with PMA/ionomycin stimulation, as PMA/Ionomycin is a calcium ionophore and EDTA is a chelating agent. No differences were found in terms of cytokine production between the unstimulated samples (Fig 3-2). Heparin collected samples had almost twice as many lymphocytes as the EDTA collected samples. Importantly, the frequencies of all three cytokines (IFN-γ, IL-2 and TNF-α) were virtually undetectable in the stimulated EDTA blood but were present in high levels in the heparinised samples for both CD4 and CD8 T cells (Fig 3-3 and 3-4).

A



B

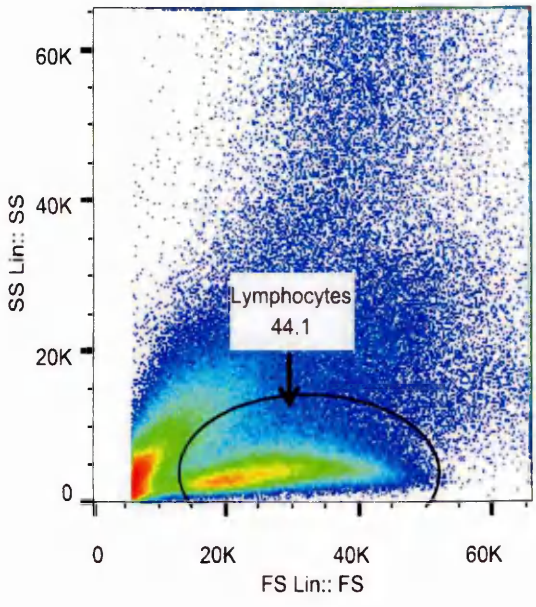


Figure 3-1: Lymphocyte population from PMA stimulated samples. A) EDTA collected sample B) Heparin collected sample; Heparin collected tubes produced twice the amount of lymphocytes than EDTA collected tubes. Gates indicate represent lymphocyte population as shown by the arrows, higher lymphocyte population in PMA/ionomycin stimulation probably due to overstimulation of these cells

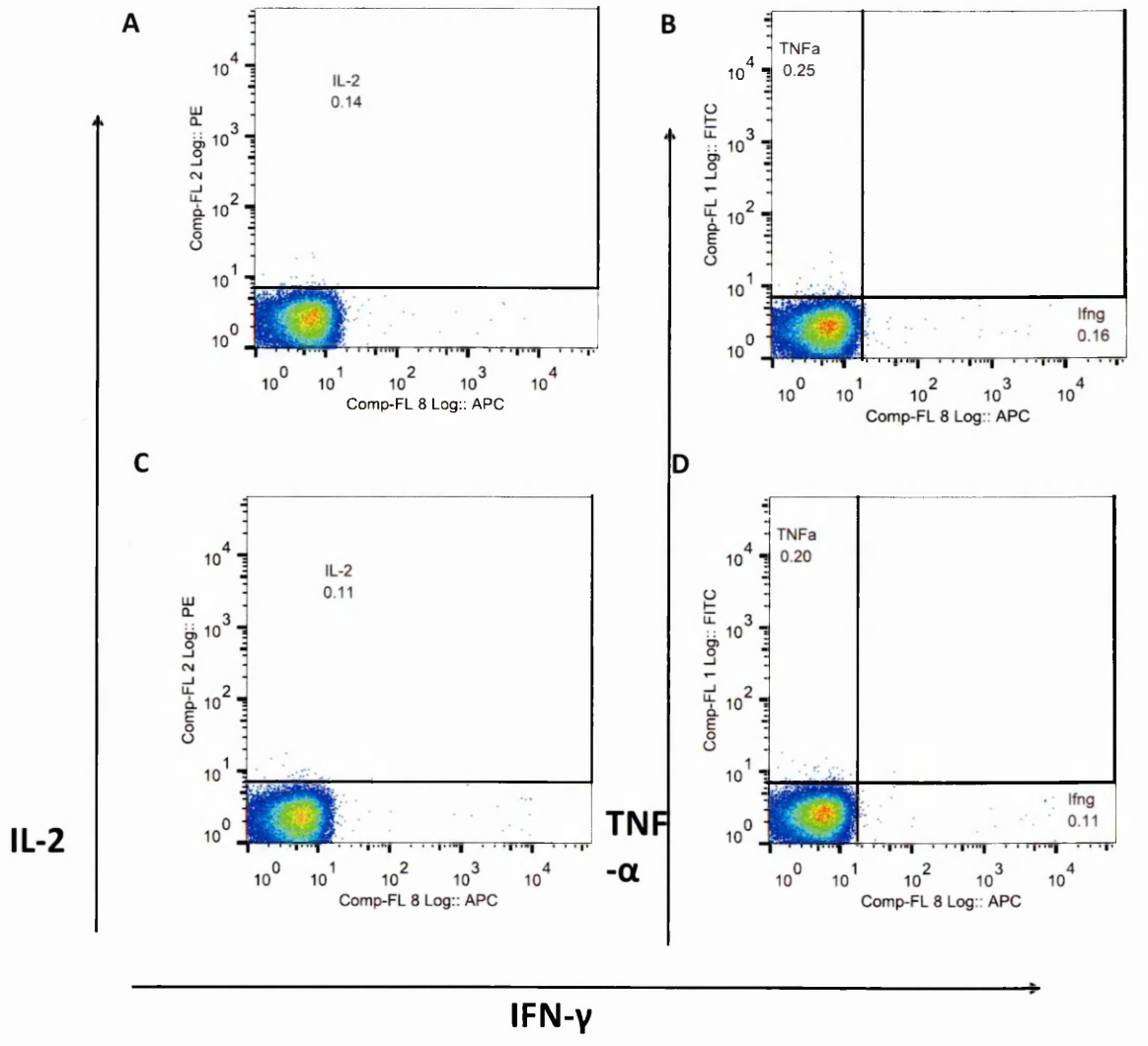


Figure 3-2: Cytokine responses from unstimulated EDTA samples (A and B) and Heparin collected samples (C and D); A and C, there was no difference in the IL-2 population from samples collected from both tube types; B and D, IFNγ and TNFα responses were not different between samples collected from both types of tubes. Plots are representative of the two individuals used for optimizations.

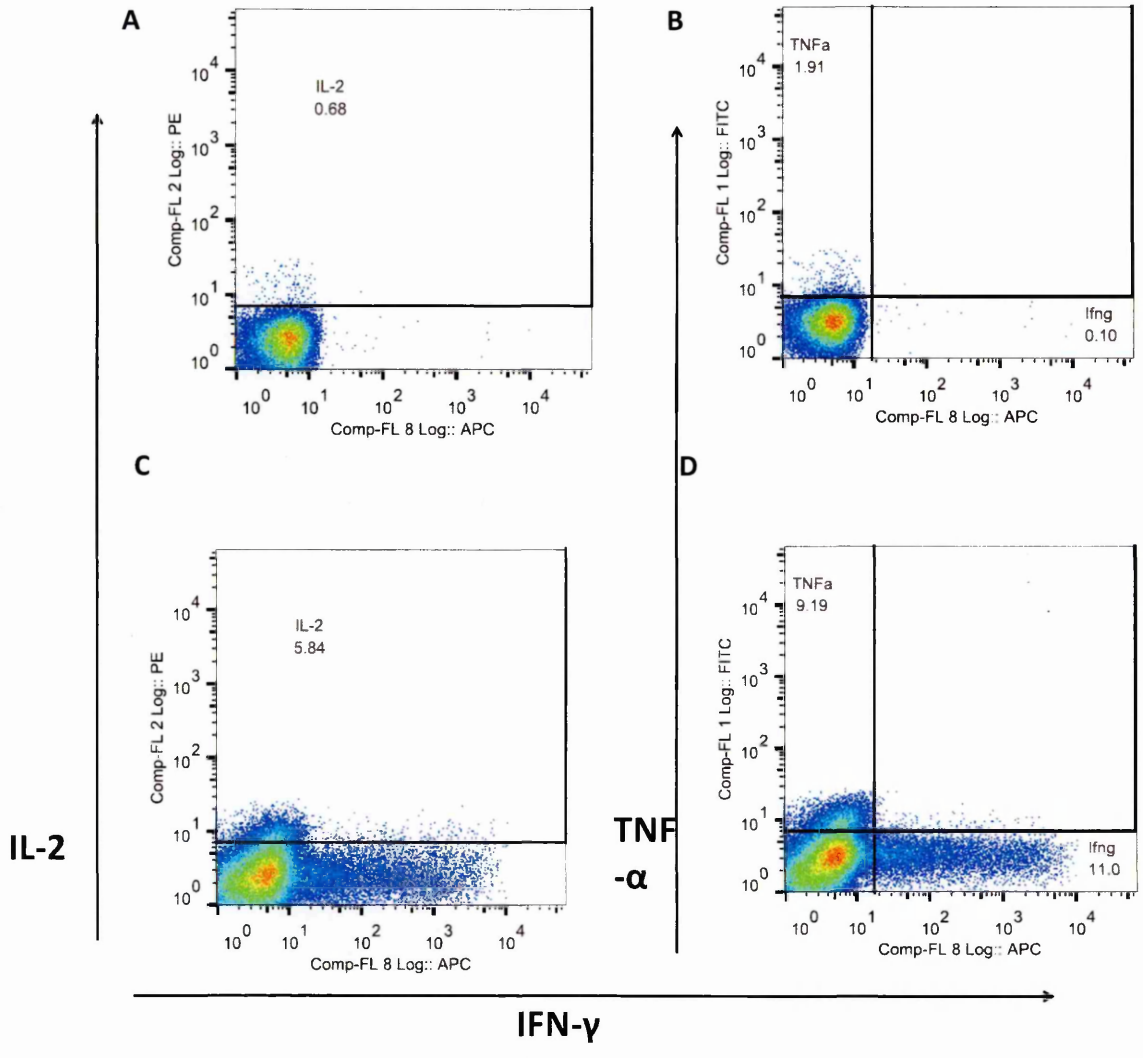


Figure 3-3: Cytokine responses generated by CD4 T cells in response to PMA/ionomycin in the same individual: A and B) responses from EDTA sample; C and D) responses from Heparinised samples; A and C) IL-2 responses were much higher in the heparin collected samples than EDTA collected samples, B and D) TNFα and IFNγ were much higher in the heparinised samples than the EDTA samples

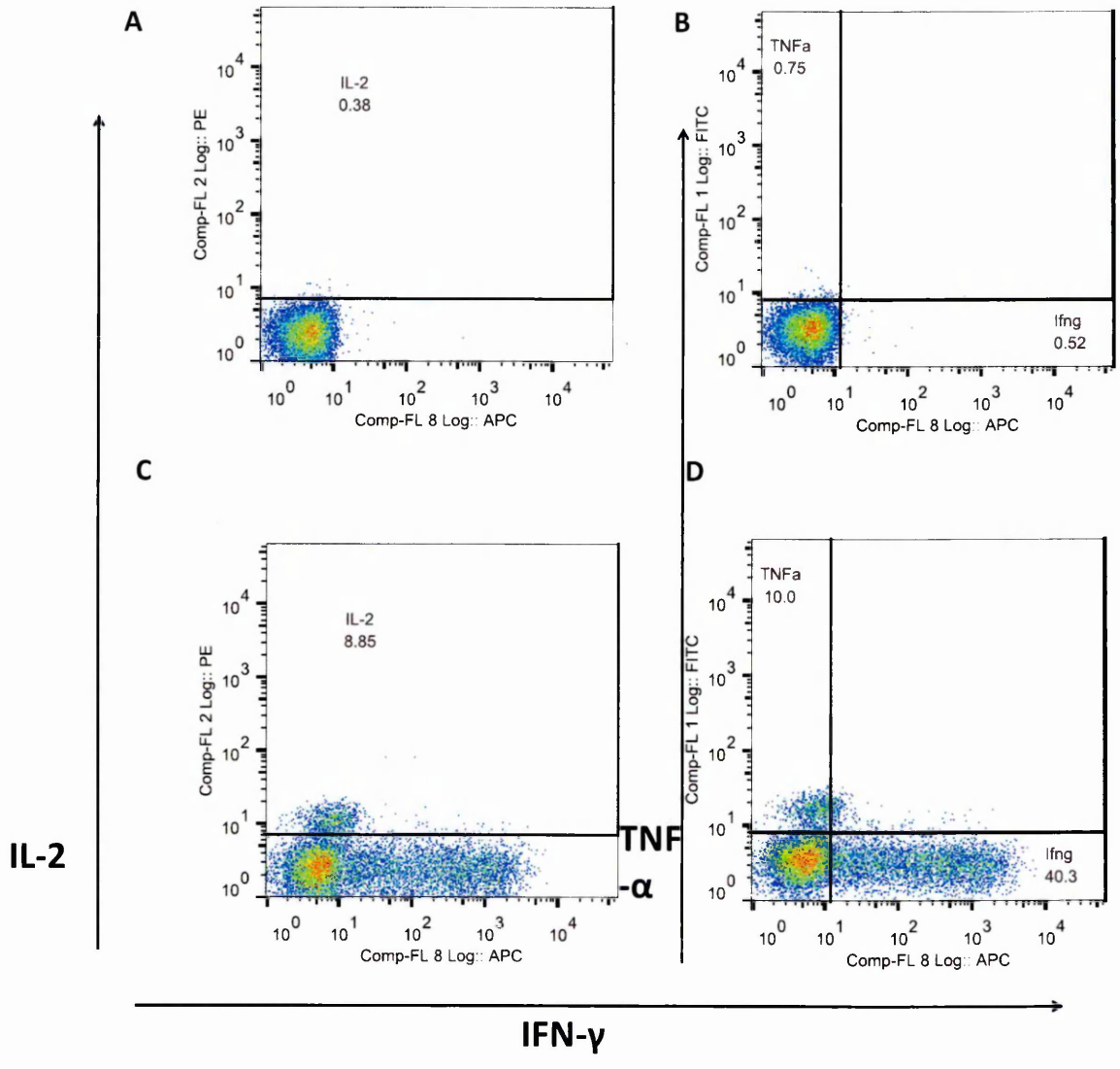


Figure 3-4:CD8 T cell cytokine response after PMA/ionomycin stimulation: A and B) EDTA samples did not produce a lot cytokines; whilst C and D) Heparin collected tubes produced as much as 10 times more cytokines than the EDTA samples . These differences might have been due to the effect of Ionomycin and EDTA on Calcium ions

3.4.2 PPD-induced cytokine responses higher in supernatants from fresh PBMCs than EDTA whole blood.

Since all blood samples were collected in EDTA as per the original study design, we could not use the whole blood stimulated samples for cytokine analysis. However, we had PBMCs stored for most of the study subjects (and thus EDTA washed off during the PBMC separation procedure). Thus, we next wanted to compare our PBMC supernatant responses with the frozen supernatants obtained from antigen-stimulated whole EDTA-blood (previously collected from the study subjects) to determine whether frozen PBMC would respond well to stimulation. These were compared to fresh PBMC. Responses to PMA/Ionomycin were highest amongst fresh cells and thawed cells with very little or no response seen from EDTA samples (Fig. 3-5). Stimulation with PPD induced significantly higher levels of IL-1 in heparinised blood from adults or frozen PBMC from the study subjects ($p=0.0055$; Fig. 3-6a). IL-10 (Th2/regulatory cytokine) levels were highest in heparinised samples and comparable for thawed and fresh cells (p-value 0.0079; Fig 3-6b ii), IL-4 production was more abundant in PBMC than whole blood stimulated cultures (Fig. 3-6b i). Cytokine responses were significantly higher from thawed cells compared to EDTA-cultures, particularly for TNF-α and IL-10 (Fig 3-6c ii). These results indicate that frozen PBMC, whilst not as optimal as fresh cells, would still provide strong cytokine responses following stimulation with our antigens of interest and were significantly better than our stored EDTA-blood samples. Despite the fact that IFNγ levels after PMA stimulation were low the PPD levels were quite normal, suggesting a detrimental effect of PMA stimulation on cryopreserved cells probably due to high polyclonal stimulation of the already attenuated cells.

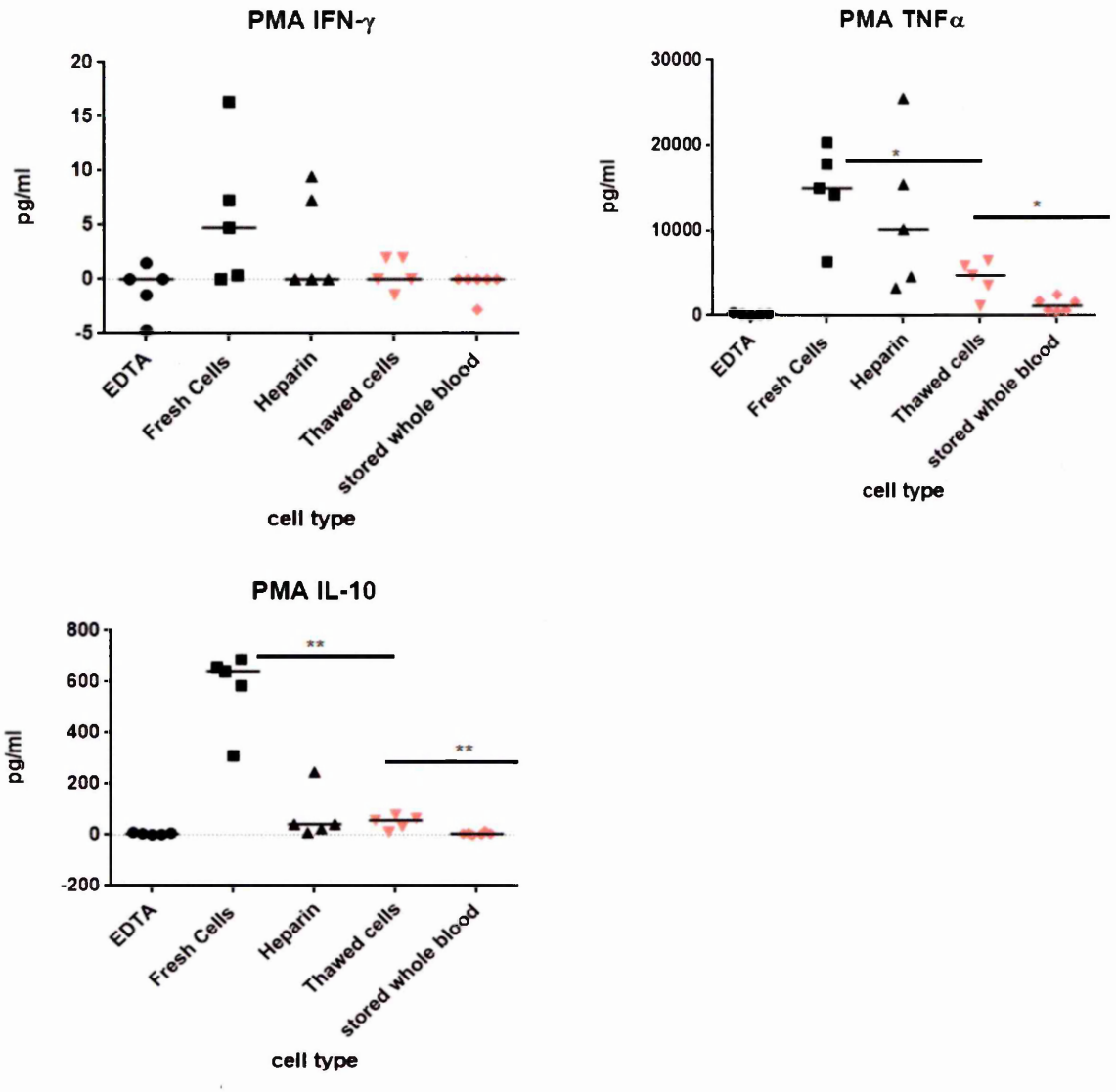


Figure 3-5: PMA/ionomycin induced cytokine responses after overnight cultures. Samples were collected adults in EDTA tubes, whole blood and PBMCs were cultured overnight, cryopreserved cells from study s collected and cultured overnight. These were compared with study supernatants collected after overnight cu = study subjects; black = healthy adult donors. Mann Whitney test; * p-value <0.05; ** p-value <0 representative of five study subjects and five healthy adults.

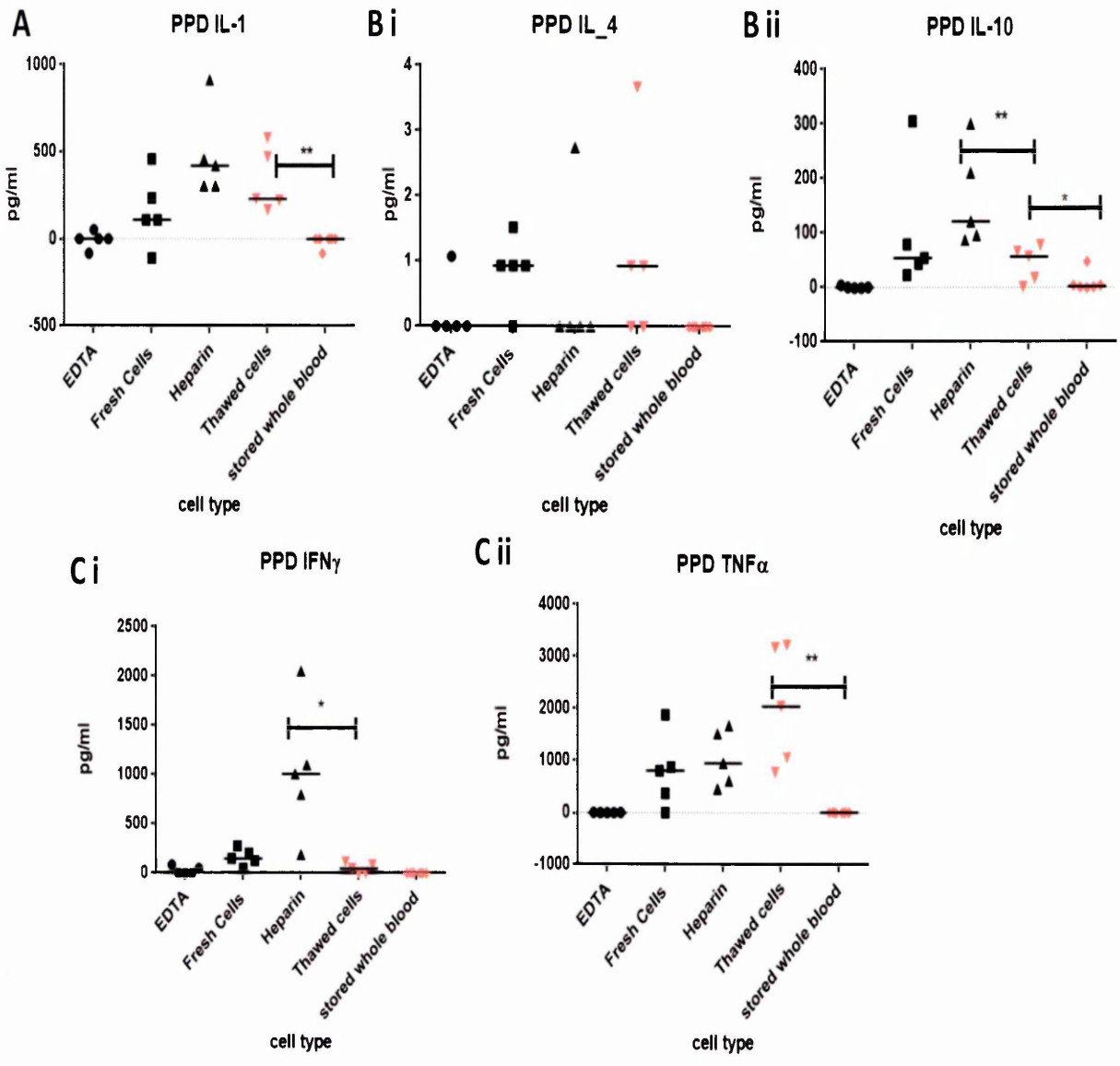


Figure 3-6: PPD induced cytokine production from different cell types collected from adults and infants, whole blood from EDTA and Heparin tubes were cultured overnight together with Fresh PBMCs collected from healthy adult donors, and cryopreserved cells from study subjects. Supernatants were analysed on the Bioplex-200 system with supernatants previously stored from study subjects after whole blood overnight cultures a) innate cytokine production b) Th2 cytokine production c) Th1 cytokine production; black- supernatants from adults; peach- supernatants from infants; Kruskal-Wallis test was used to analyze data generated. * P-value <0.05; ** p-value <0.01

3.5 Discussion

The original study design for this project involved collecting blood from study subjects into EDTA vacutainers followed by overnight stimulation with specific and non-specific antigens. Stimulated cultures were stored at -70°C (for ICS) or -20°C (for Luminex) prior to analysis. However, we soon discovered that EDTA is a chelating agent in addition to its anticoagulation properties and therefore is not recommended for whole blood stimulation assays. We therefore needed to determine if cryopreserved cells taken from the same subjects could be used instead. Importantly, we were able to show that cryopreserved cells can be used for stimulation experiments, inducing high levels of cytokines in response to stimulation. Furthermore it also meant we could control for inter-assay variability by allowing us to assay study subjects with blood samples from each visit simultaneously.

Ca^{2+} has been shown to play an important role in T cell signalling before antigen independent signalling begins (Feske, 2007). We showed that stimulated whole blood collected in heparin tubes had a better cytokine response than whole blood collected in EDTA tubes. EDTA works by inhibiting the Ca-flux thus resulting in impaired responses to antigen stimulation. We showed that $0.1x10^6$ cells were sufficient to generate significant amounts of cytokines after stimulation. Thawed cells from study subjects produced higher amounts of cytokines compared to their EDTA-whole blood stimulated counterparts from the same subjects. Thawed cells were able to produce almost the same amount of cytokines as the fresh cells even though they were from different populations (adults and infants) that are at completely different stages of immune development.

Heparinised samples produced more cytokines in response to PPD than their EDTA counterparts from the same subjects, in concurrence with the results from the flow cytometric data. Heparinised whole blood samples collected from adults were the only ones that showed production of IFN-γ in response to PPD, although some response could be seen from the fresh cells collected from adults these were lower in the thawed cells

collected from the infants. An explanation for this might be that the whole blood samples contain a diverse milieu of cell types some of which are capable of producing IFN-γ responses and the PBMCs contain predominantly lymphocytes, thus production from one type of cell would be expected to be lower than in a sample with a mix of cells. PPD induced cytokine production was assessed and this response may be affected by BCG vaccination and also exposure to non-tuberculous mycobacteria (NTM). This might be responsible for the high cytokine responses in heparin collected samples and fresh PBMCs for some of the cytokines as adults were not only exposed but have also been BCG-vaccinated.

In conclusion, we demonstrated significant cytokine responses using cryopreserved cells, thus allowing the study to continue with this modification. For future studies we determined that down-stream analysis of cytokine in supernatants and by ICS cannot be done using EDTA samples.

Chapter 4: Analysis of soluble cytokine profiles induced by BCG vaccination

4.1 Introduction

Bacillus Calmette-Guérin (BCG) vaccination induces protection against childhood TB as well as heterologous protection against unrelated pathogens such as *Candida albicans* and *Staphylococcus aureus* and regression of bladder cancer (Alexandroff et al., 1999; Kleinnijenhuis et al., 2012). Protective correlates of BCG vaccination remain largely unknown hence immunogenicity based on the induction of cytokines, particularly IFN-γ, is commonly used as a measure of vaccine efficacy. This chapter describes mycobacterium-specific and heterologous responses induced by BCG using longitudinal analysis of multiplex cytokine profiles in plasma and antigen-stimulated culture supernatants of vaccinated and unvaccinated infants. The overall aim of this work was therefore provide further insight into mycobacterial and non-mycobacterial protective immunity induced by BCG.

4.1.1 Induction of an innate immune response after BCG vaccination

The innate immune system is diverse and comprises a variety of cells including NK cells, neutrophils, macrophages and dendritic cells. It is critical at every stage of human development as it provides rapid protection against invading pathogens before the generation of an adaptive immune response. Genetically conserved pattern recognition receptors (PRRs) such as Toll-Like receptors (TLRs) recognise pathogen associated molecular patterns (PAMPs) and are essential for microbial recognition by macrophages and dendritic cells (Medzhitov & Janeway, 1997; Medzhitov et al., 1997). PRR signaling induces proinflammatory cytokine and chemokine production, phagocyte recruitment, complement activation and APC mobilization (West, Koblansky, & Ghosh, 2006). The importance of TLR ligands in the immune response to bacteria was shown by the use of TLR ligands as adjuvants in vaccination of mice, resulting in much better induction of Th1 responses (Iwasaki & Medzhitov, 2004). In addition, mice deficient in Myeloid Differentiation 88 (MyD88) were inefficient in Th1 differentiation but instead generated Th2

responses (Schnare et al., 2001;Kaisho et al., 2002). Age-dependent differences in responses to TLR stimulation have been observed in humans with an early bias toward Th2-polarising cytokines leaving the neonate susceptible to microbial infections and contributing to vaccine response impairment (reviewed by Levy, 2007; Belderbos et al., 2009; Burl et al., 2011). BCG vaccination at birth has been shown to induce high levels of Th1 cytokines such as IFN-γ (Marchant et al., 1999; Ota et al., 2002; Vekemans et al., 2001), thus altering the balance between the Th1 and Th2 responses and possibly preventing allergy development down the line. One aim of this chapter was to determine if TLR ligands could induce differential cytokine profiles in vaccinated compared to unvaccinated infants.

4.1.2 Role of adaptive cytokines in BCG immunogenicity

Cytokines are cell signaling molecules that regulate different immune cell functions such as proliferation, apoptosis and antibody production, as well as stimulating cell recruitment to sites of inflammation, infection and trauma. Cytokines are important in the differentiation of naive T cells into different cell subsets: IL-12 drives T helper (Th)1 differentiation whilst IL-4 drives Th2 differentiation (Akira, Takeda, & Kaisho, 2001); IL-10 and IL-13 together with IL-4 activate B cells, whilst IFN-γ activates the cytotoxic T cell pathway (Dinarello, 2000) and innate cells (Soudja et al., 2014). Several studies have demonstrated the induction of robust IFN-γ, IL2 and TNF-α responses after BCG vaccination (Marchant et al., 1999; Vekemans et al., 2001; Ota et al., 2002; Burl et al., 2010; Kleinnijenhuis et al., 2012), while Th2 cytokines, although produced after vaccination, are induced at a much lower magnitude (Ota et al., 2002; Soares et al., 2008). IL-17 has also been shown to be induced in neonates after BCG vaccination (Burl et al., 2010; Kagina et al., 2010; Li et al., 2012). Despite all these effects, immune correlates of protection against TB are yet to be properly defined.

4.1.3 Heterologous and sex differential effects of BCG against unrelated pathogens and diseases

A meta-analysis of clinical trial data of the effects of BCG vaccination from the US and UK in the 1940s and 50s estimates a 25% reduction in mortality from diseases other than TB with a 95% CI of 6-41% (Shann, 2010). Recent randomized trials in low-birth weight neonates indicated that BCG vaccination reduces neonatal mortality in this group by up to 17% with a mortality rate ratio of 0.83 (0.63-1.08) (Aaby et al., 2011). BCG is being used in Brazil to protect household contacts of leprosy, which is caused by *Mycobacterium leprae*, a meta-analysis of experimental data has indicated that BCG vaccination offers an average of 26% protection against leprosy (95% CI 14-37%) whilst observational studies suggest a 61% protective effect (95% CI 51-70%) (Setia et al., 2006). A decrease in fungal burden in the kidney together with better survival rates was observed in BCG vaccinated mice after *Candida albicans* inoculation (Kleinnijenhuis et al., 2012). Apart from its effectiveness against other unrelated infectious pathogens/diseases; it has been shown to be effective in its use as a therapeutic against bladder cancer after repeated instillations in the bladder (Alexandroff et al., 1999). Interestingly, a lot of the effects of BCG vaccine appear to be sex-differential and this has also been shown with other vaccines. For example, girls that received diphtheria, tetanus and pertussis (DTP) and hepatitis B vaccine virus HBV in rural Gambia had a much higher mortality rate when compared to males (Aaby, Jensen, & Walraven, 2006), while the BCG response was strongly related to survival (all-cause mortality) in girls when compared to boys in Guinea Bissau (Roth et al., 2006). Clearly there is a need for further investigation into the heterologous protective effects against non-mycobacterial infections, and sex-related differences in regards to the BCG vaccines. These data will help determine the optimal vaccine schedule and will enable health care workers to know the benefits of giving BCG alone or in combination with other vaccines.

This chapter describes longitudinal changes in *ex-vivo* cytokine profiles in plasma and *in vitro* cryopreserved PBMC-stimulated culture supernatants in BCG-vaccinated compared to unvaccinated subjects. Responses to expanded programme on immunization (EPI) vaccine-specific and unrelated antigens were analyzed as well as to TLR agonists, to determine the impact of BCG vaccination on infant immunity and to increase our knowledge of protective markers induced by BCG.

Hypothesis:

- Infants vaccinated at 6 weeks would have higher cytokine immune responses to Toll-Like receptor agonists (naïve responses) and heat killed unrelated pathogens (heterologous responses)

Aims:

- Examine longitudinal changes in ex-vivo and in vitro cryopreserved PBMC-stimulated culture supernatants in BCG vaccinated subjects compared to unvaccinated subjects
- Measure cytokine responses to mycobacterial antigens, EPI vaccine specific responses, as well as TLR agonists.

4.2 Methods

4.2.1 Overnight culture conditions

Peripheral Blood Mononuclear cells (PBMCs) were thawed and rested for 6 hours as described in Chapter 2. 100,000 cells were stimulated per condition for 10 different conditions in 100µL of culture medium overnight for 16 hours at 37°C 5%CO_2. The culture conditions were: medium only (negative control), PMA/ Ionomycin (Positive control), Purified protein derivative (PPD) and Tetanus Toxoid (TT) (vaccine specific responses), Lipopolysaccharide (LPS, TLR4), Heat killed *Listeria monocytogenes* (HKLM, TLR2) and CLO-75 (TLR 7/8) (innate agonists) and heat killed unrelated pathogens (*Candida albicans, Streptococcus pneumoniae, and Escherichia coli* (kind gift of Mihai Netea,

Radbound University Medical Centre, Nijmegen, Netherlands)). After the overnight stimulation, 100µL of culture medium was added; samples were spun at 3000 rpm for 10 minutes, supernatants were collected and stored at -20°C.

4.2.2 Cytokine analysis via multiplex bead array

Samples were analyzed as described in Chapter 2. Briefly, 50µL of samples were added onto beads (diluted 1 in 2 with buffer) coupled with the cytokines of interest (IFN-γ, TNF-α, IL-2, IL-4, IL-17, IL-10 and IL-12) and incubated for 30 mins. Biotinylated secondary antibody (diluted 1 in 2 with buffer) was added and another 30 minute incubation at room temperature done. Streptavidin-PE was added, samples incubated for 10 mins and results read on the Bioplex-200 systems using Bioplex manager v4.0 software (Bio-Rad, Belgium).

4.2.3 Statistical analysis

The Heat map was generated on excel after subtraction of negative median values from median values of antigen stimulated samples. Conditional formatting was then carried out based on percentiles, lower percentiles, mid percentiles and high percentiles. All statistical analysis was done using GraphPad Prism V6 following background subtraction. Mann–Whitney U test was carried out to test between two groups and Kruskal-Wallis test used to test between three groups. A p-value less than 0.035 was considered significant after adjusting for multiple comparisons (q-value).

4.3 Results

4.3.1 *Ex-vivo* plasma cytokine levels

The underlying cytokine milieu in BCG vaccinated compared to unvaccinated infants was determined by analyzing plasma cytokine levels. Type I cytokines (IL-2, IL-12 and IFN-γ) were higher than Th2 cytokines in all subjects and increased over time in both groups (Fig 4-1A-B), IL-4 levels increased steadily in both groups, whilst IL-10 increased significantly in the unvaccinated from 1 week post-vaccination to 12 week post-vaccination (Fig 4-1C)

but did not increase in the vaccinated group. IL-17 levels however, decreased in both groups at 1 week post-vaccination and increased at 12 week post-vaccination (Fig 4-1 D). There were no differences observed between the two groups at any time point in terms of plasma cytokine levels.

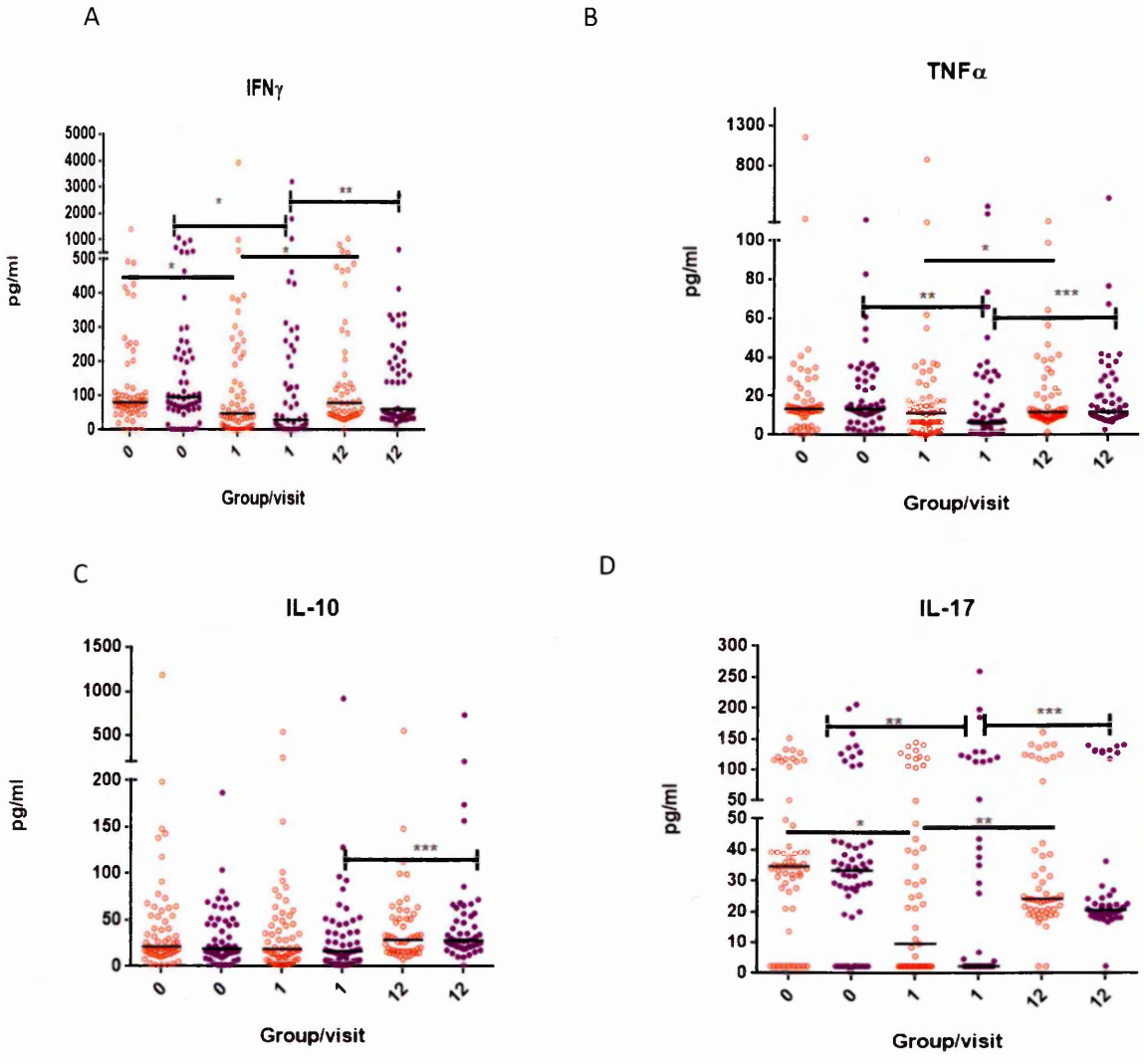


Figure 4-1: Ex-vivo cytokine levels in plasma of BCG vaccinated and unvaccinated infants. BCG vaccinated and naive subjects had variable Th1, Th2 and Th17 cytokine levels at 0 (pre-vaccination/ 6 weeks), 1 (1-week post-vaccination/7 weeks) and 12 (12 weeks post-vaccination/18 weeks). Closed purple circles represent unvaccinated and open red circles represent the vaccinated group. *- q value ≤0.035, **- p≤ 0.01, ***p< 0.0001.

4.3.2 Cytokine production after antigenic stimulation

4.3.2.1 Global responses

A heat map was generated in order to obtain a global picture of cytokine profiles (Fig 4-2). Median levels of IL-2, IL-4 and IL-12 were relatively low (blue) following all stimulations, while TNF-α, IFN-γ and IL-10 were relatively high (red). There appeared to be little or no reactivity to TT and reactivity to PPD (used as a marker of BCG vaccine-related responses) induced surprisingly low levels of IL-10 and IFN-γ even in the vaccinated group. When the fold-change for each cytokine was analyzed at the different time-points, the BCG-vaccinated group had higher Th1 increases than the unvaccinated group after PPD stimulation (IFN-γ: 1 week post-vaccination -12 weeks post-vaccination = 5 fold and 2-fold respectively; TNF-α: baseline-12 weeks post-vaccination = 25 fold and 7-fold respectively). In contrast, but in-line with our data discussed in the previous section, the unvaccinated group had higher IL-10 fold changes (74-fold compared to 5-fold for the vaccinated group). In response to TLR agonist stimulation there was a general decrease in all cytokine levels at 1 week post-vaccination but an increase again by 12 weeks post-vaccination in the vaccinated, whilst an increase at 1 week post-vaccination was observed in the unvaccinated but this decreased at 12 weeks post-vaccination. Heat killed *S. pneumoniae* and *E. coli* also produced IFN-γ, TNF-α and IL-10 responses, with higher IFN-γ and TNF-α in the unvaccinated infants, and the vaccinated group had lower IL-10 responses. Background values were high for IFN-γ and TNF-α suggesting spontaneous production, most likely from innate cells.

		BCG-Unvaccinated							BCG-Vaccinated at 6-weeks						
Antigen	Visit	IL-2	IL-4	IL-10	IL-12p70	IL-17	IFN-γ	TNF-α	IL-2	IL-4	IL-10	IL-12p70	IL-17	IFN-γ	TNF-α
PMA	V1	159	0	2	0	48	18	142	114	1	0	4	20	76	253
	V2	106	0	2	0	43	39	124	189	1	0	3	34	60	188
	V3	111	1	46	3	83	26	189	151	2	38	5	86	62	440
PPD	V1	0	0	1	0	0	0	10	0	0	2	0	0	0	1
	V2	1	0	17	0	1	14	48	0	0	1	0	0	0	5
	V3	0	0	74	0	0	7	70	1	0	10	0	0	5	25
TT	V1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V3	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Clo-75	V1	5	3	85	10	55	159	2603	1	3	49	11	21	95	1177
	V2	9	2	174	28	45	195	2927	1	1	12	2	4	24	448
	V3	8	5	145	19	89	235	2943	7	2	48	17	37	155	1604
HKLM	V1	2	1	167	3	8	61	663	1	1	87	4	17	42	668
	V2	7	2	207	6	34	143	1373	1	1	12	0	0	24	76
	V3	3	2	242	1	27	103	1432	4	1	94	1	12	49	647
LPS	V1	0	1	176	15	5	82	721	2	3	116	15	38	78	740
	V2	6	2	247	21	32	89	1626	0	0	26	3	0	3	99
	V3	2	1	217	7	14	65	676	2	1	67	4	2	51	358
SP	V1	0	0	22	0	0	16	175	0	0	13	1	0	0	13
	V2	0	0	48	1	0	17	113	0	0	2	0	0	0	2
	V3	0	0	86	0	0	0	34	0	0	18	0	0	10	111
CA	V1	0	0	0	0	0	0	2	0	0	0	1	0	0	5
	V2	0	0	1	0	0	5	2	0	0	0	0	0	0	1
	V3	0	0	19	0	0	0	5	0	0	0	0	0	6	33
EC	V1	1	0	109	1	0	13	651	0	1	33	6	12	43	81
	V2	6	1	221	16	14	100	668	1	0	11	4	1	5	72
	V3	2	1	264	8	17	41	987	2	1	60	6	2	53	613
NIL	V1	5	2	33	12	40	95	100	2	2	15	3	2	49	49
	V2	4	3	41	13	45	89	101	4	3	29	12	53	84	65
	V3	6	3	68	13	39	131	290	5	3	30	16	51	97	146

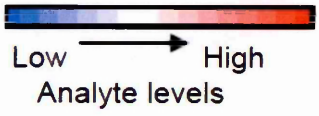


Figure 4-2: Heat map showing median levels after background subtraction for various stimulations of cryopreserved PBMCs, conditional formatting was conducted based on percentile with blue being low percentile, white mid percentile and red high percentile separately for each stimulus. Baseline (6 weeks/ pre-vaccination), 7 weeks/ 1 week post-vaccination, 18 weeks/12 weeks post-vaccination, data is representative of all study subjects.

4.3.2.3 Cytokine production in response to a polyclonal stimulator is not affected by BCG vaccination

Following PMA/Ionomycin stimulation, Th1 and Th17 cytokine responses were higher than anti-inflammatory responses, and levels were comparable between the vaccine groups indicating that any observed differences in response to specific antigens was not due to differences in the ability of subjects to respond to mitogenic stimulation (Fig 4-3). We did have some subjects that had low responses to PMA, this might be due to an effect of too much polyclonal expansion on already weakened/ attenuated cells, and despite this we did see responses to PPD.

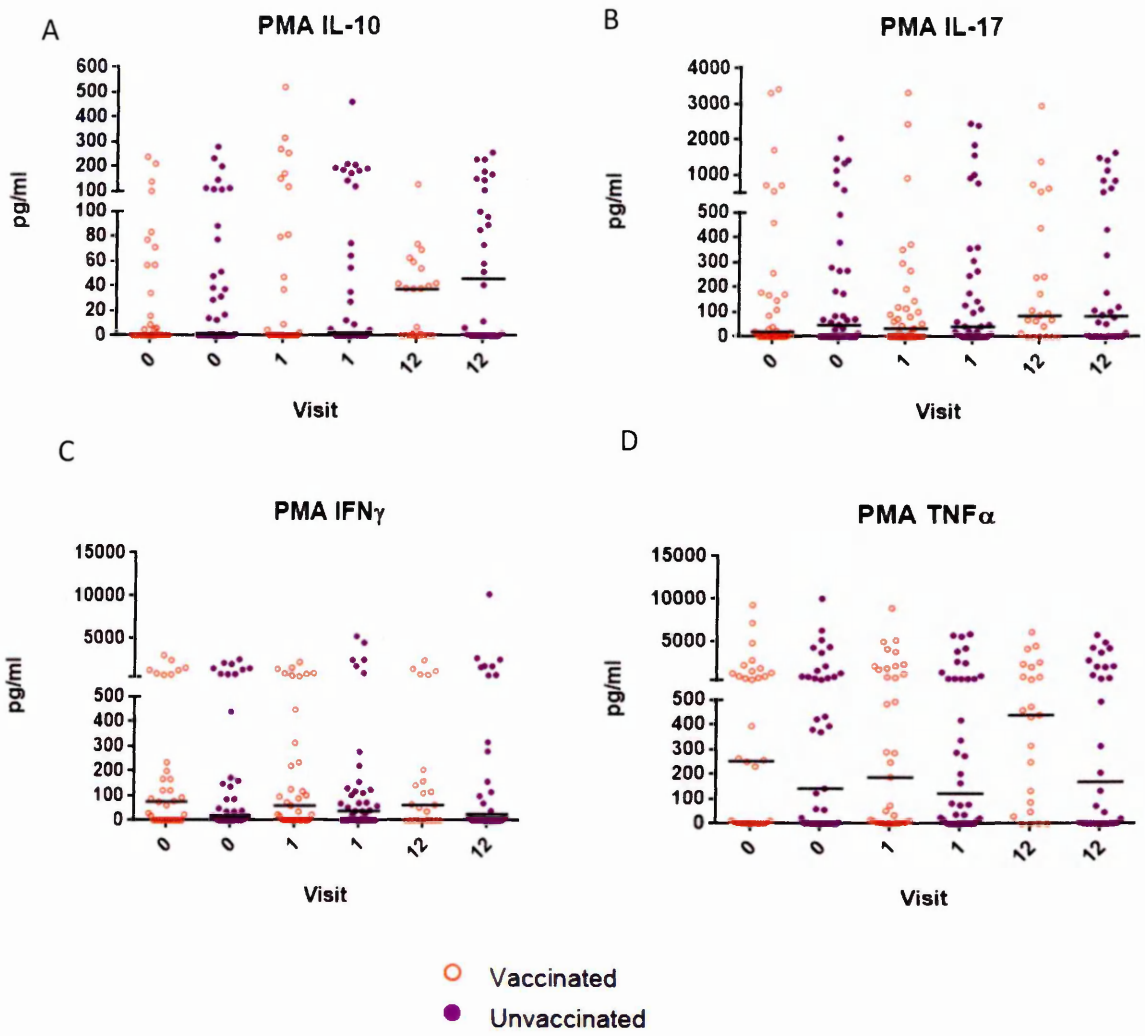


Figure 4-3: Cytokine responses after polyclonal (PMA/Iono) stimulation. Red open circles represent vaccinated group, closed purple circles represent the unvaccinated group, 0 (pre-vaccination/6 weeks), 1 (1 week post-vaccination/7 weeks) and 12 (12 weeks post-vaccination/18 weeks).The no. of subjects at the different time points were, time point (time since vaccination/age of infant)no. of subjects-vaccinated, unvaccinated: 0(baseline/ 6 weeks)-34 vaccinated subjects,41 unvaccinated infants; 1(1 week post vaccination/ 7 weeks)37 vaccinated, 40 unvaccinated; 12 (12 weeks post-vaccination/ 18 weeks)23 vaccinated, 30 unvaccinated subjects.

4.3.2.4 Differential cytokine profiles in response to BCG -specific antigens

In order to measure vaccine-specific responses we used responses following stimulation with PPD as a surrogate for responses to BCG. Th1 cytokine production in PPD cultures was similar in both groups despite the unvaccinated group not yet receiving BCG. IL-2 and IL-12 were low at all time-points while IFN-γ and TNF-α responses were higher generally, but not significantly different between vaccine groups. Median IL-4 levels remained low at all time-points in both groups, while IL-10 levels steadily increased over time in the unvaccinated group. IL10 levels in the vaccinated group decreased at 1-week post-vaccination but increased again by week post-vaccination and the Th1/Th2 ratio was highest at this time point. Despite the low median values observed, there was a clear cut distinction between responders (R) and non-responders (NR) in both vaccine groups in terms of cytokine production over time (Fig 4-4); however, there were no differences between the proportions of responders between the groups at any time-point using chi-square analysis.

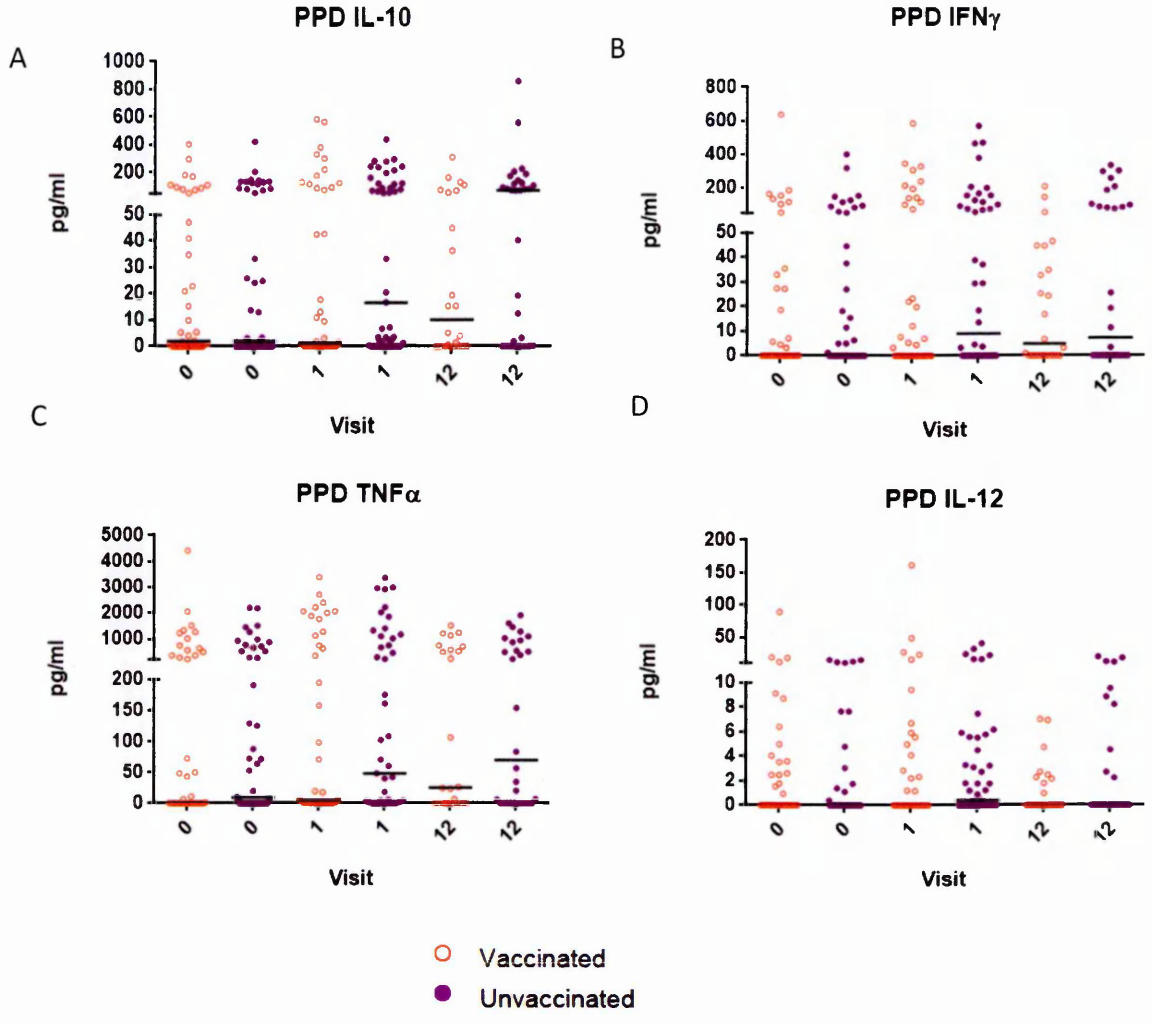


Figure 4-4: Cytokine responses after vaccine specific PPD antigen stimulation. Red open circles represent vaccinated, closed purple circles represent the unvaccinated, 0(6 weeks/ Pre-vaccination), 1 (7 weeks/ 1 week post-vaccination), 12 (18 weeks/ 12 weeks post-vaccination). Similar cytokine responses observed between the groups at the various time points. Mann Whitney U test was used to assess for differences between two groups and Kruskal Wallis test used to compare across three time points. The no. of subjects at the different time points were, time point (time since vaccination/age of infant) no. of subjects-vaccinated, unvaccinated: 0(baseline/ 6 weeks)-43 vaccinated subjects, 49 unvaccinated infants; 1(1 week post vaccination/7 weeks) 43 vaccinated, 47 unvaccinated; 12 (12 weeks post-vaccination/ 18 weeks) 24 vaccinated, 30 unvaccinated subjects.

4.3.2.5 Differential responses to TLR agonists with BCG vaccination

TLR agonists were used to measure innate immune responses, which may be useful for future vaccine development if they could be used as vaccine adjuvants. One of the interesting findings was the increase in IL-12 levels in the unvaccinated group in HKLM cultures (p=0.015) (Fig 4-5A). In addition, IL-17 levels were also higher in the unvaccinated group at 1 week post-vaccination compared to the vaccinated and responses were significantly higher after HKLM stimulation compared to the vaccinated infants (median (IQR): vaccinated=0pg/mL (0-206); unvaccinated=34pg/mL (0-424); p=0.0060) (Fig 4-5 B).

The same trends were observed for both Th1 and Th2 cytokine responses: the responses within the unvaccinated group were generally increased at 1 week post-vaccination and decreased again at 12 week post-vaccination , whilst the opposite was observed in the vaccinated group, with the early post-vaccination time-point (1 week post-vaccination) delineating the biggest differences between the groups as expected. For example there was significantly lower IL10 in the vaccinated group compared to the unvaccinated at 1 week post-vaccination after stimulation with CLO-75 (median (IQR): unvaccinated 174pg/mL (0-764), vaccinated 12pg/mL (0-855); p=0.0034) (Fig 4-5C); HKLM (median (IQR): unvaccinated 207pg/mL (0-1128), vaccinated=12pg/mL (0-843); p=0.0014) and LPS (median (IQR): unvaccinated 247pg/mL (0-1052), vaccinated=20pg/mL (0-951); p=0.0045) (Fig4-5D). Interestingly, IFN-γ after CLO-75 stimulation (p=0.0166), HKLM (p=0.0171) and LPS (p=0.0334) stimulations also significantly lower in the vaccinated group.

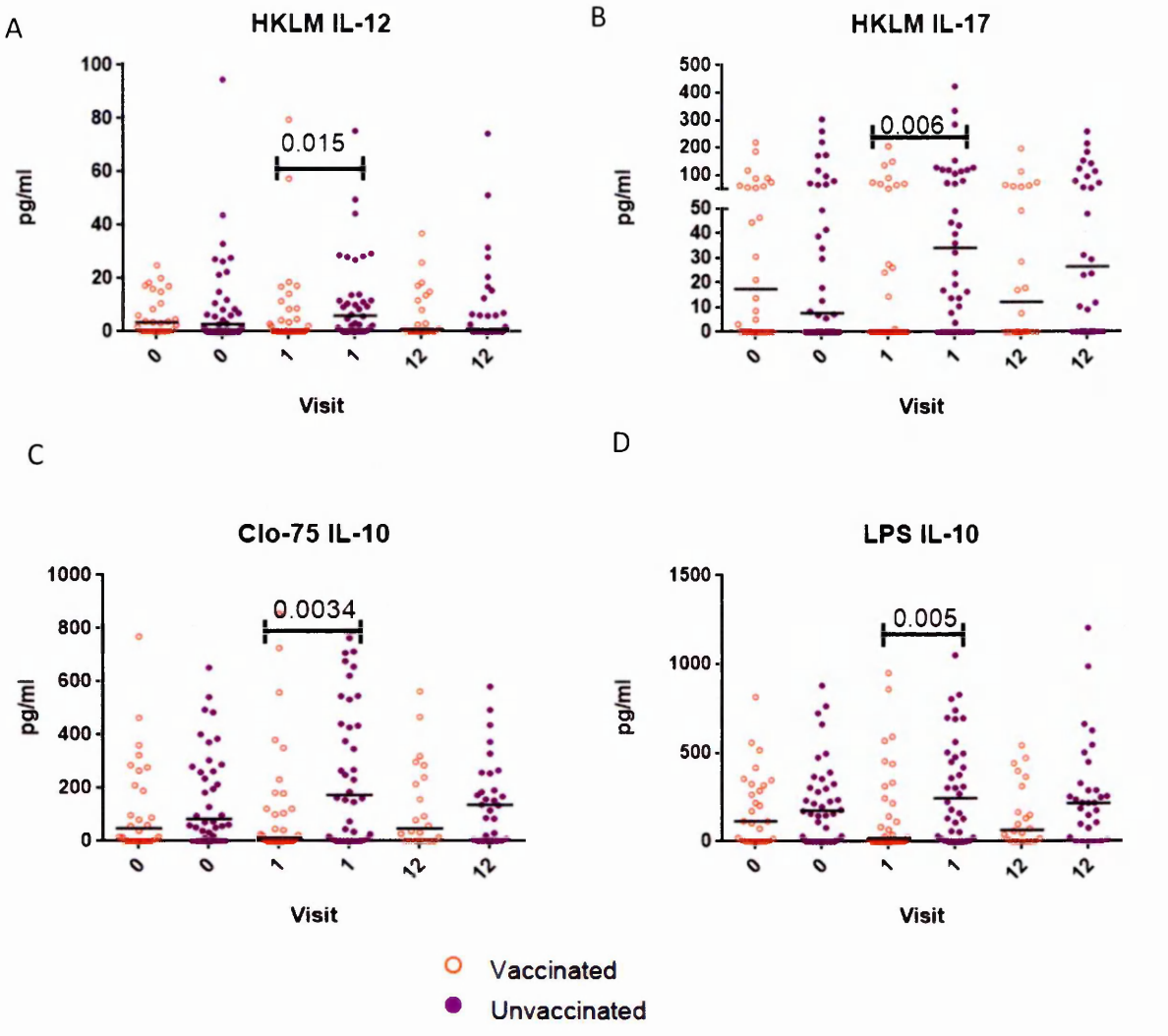


Figure 4-5: Cytokine production in response to TLR agonist stimulation in vitro. Red open circles represent vaccinated infants, closed purple circles represent the unvaccinated, 0 (6 weeks/ Pre-vaccination), 1 (7 weeks/ 1 week post-vaccination), 12(18 weeks/ 12 weeks post-vaccination). Higher cytokine production to agonists observed in the unvaccinated at 1 week post-vaccination. Mann Whitney U test was used to assess for differences across two groups and Kruskal Wallis test used to compare across three time points. The no. of subjects at the different time points were, time point (time since vaccination/age of infant) no. of subjects-vaccinated, unvaccinated: 0(baseline/ 6 weeks)-28 vaccinated subjects, 38 unvaccinated infants; 1(1 week post vaccination/7 weeks) 35 vaccinated, 38 unvaccinated; 12 (12 weeks post-vaccination/18 weeks) 22 vaccinated, 30 unvaccinated subjects.

4.2.2.6 Lower IL-10 reactivity to unrelated pathogens in BCG-vaccinated infants

We hypothesized that BCG vaccination may enhance responses to unrelated common pathogens; hence we measured cytokine production after stimulation of PBMCs with heat killed *Streptococcus pneumoniae* (SP), *Candida albicans* (CA) and *Escherichia coli* (E co). There were very few differences seen between the groups with the most striking difference being lower IL-10 levels in the vaccinated compared to unvaccinated group after E co stimulation at 1 week post-vaccination (median (IQR): 221pg/mL (0-1367) compared to 11pg/mL (0-1522) in the vaccinated group; p=0.0185) (Fig 4-6A). TNF-α, IL12, IFN-γ and IL17 showed some differences depending on the vaccine group and time-point but none were significantly different (Fig 4-6B-C).

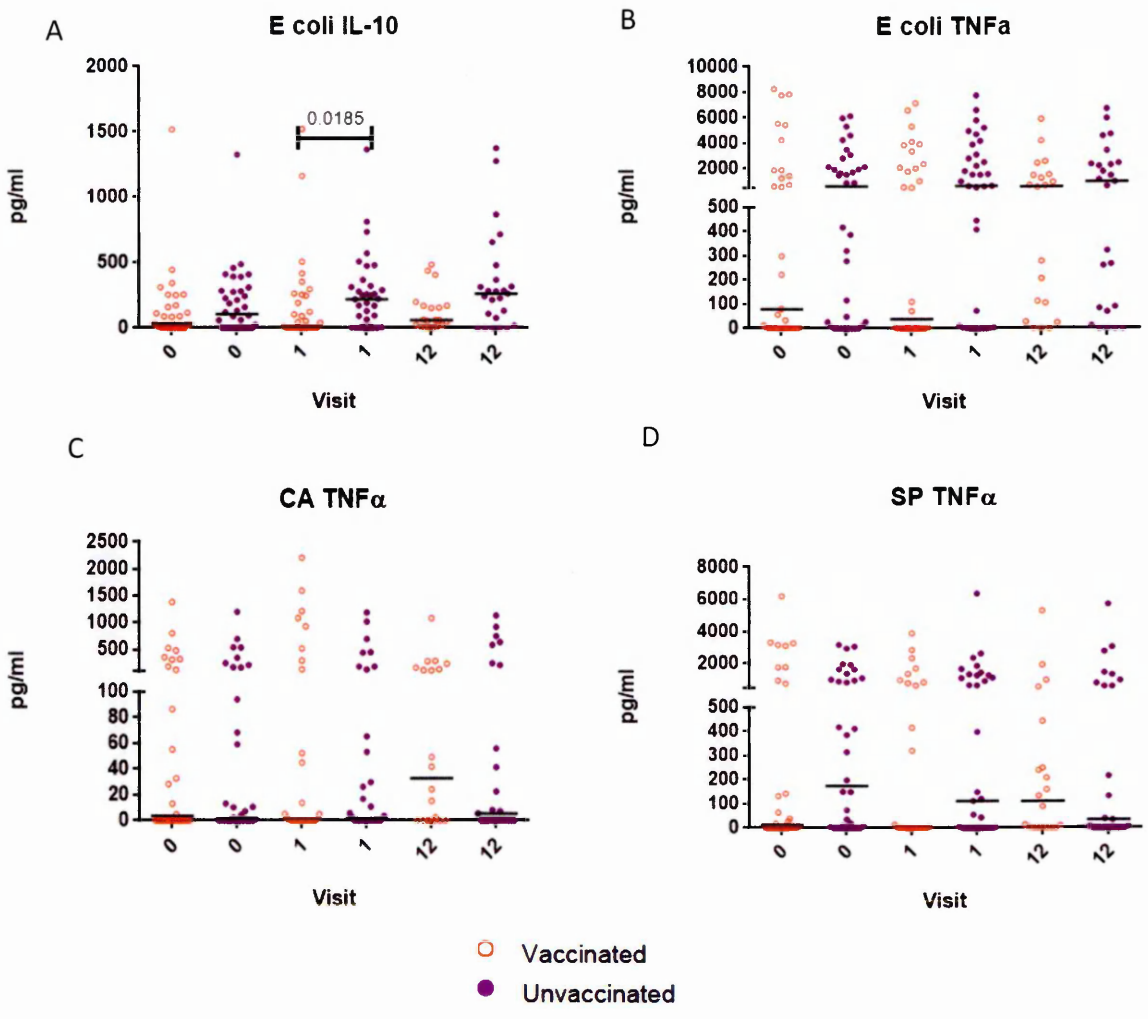


Figure 4-6: Cytokine production after stimulation with heterologous antigens. Red open circles represent vaccinated infants, closed purple circles represent the unvaccinated, 0 (6 weeks/ Pre-vaccination), 1 (7 weeks/ 1 week post-vaccination), 12 (18 weeks/ 12 weeks post-vaccination). Similar responses were observed between the two groups, the unvaccinated had higher IL-10 levels post vaccination after *E. coli* stimulation. Mann Whitney U test was used to assess for differences between two groups and Kruskal Wallis test used across three time points. The no. of subjects at the different time points were, time point (time since vaccination/age of infant) no. of subjects-vaccinated, unvaccinated: 0(baseline/6 weeks)-31 vaccinated subjects, 36 unvaccinated infants; 1(1 week post vaccination/7 weeks)33 vaccinated, 35 unvaccinated; 12 (12 weeks post-vaccination/18 weeks)20 vaccinated, 25unvaccinated subjects.

4.3.2 Vaccinated females had lower IL-10 levels compared to unvaccinated females after unrelated pathogen and TLR agonist stimulation

It has been shown that responses to vaccines may be gender-dependent, thus all responses were analyzed based on gender in our study groups. No differences were seen after PPD stimulation, but after stimulation with *E coli* and SP, lower IL-10 levels were seen in the vaccinated compared to unvaccinated females (Fig 4-7A); *E coli* (vaccinated 2.4(0-1162) pg/mL; unvaccinated 249.4(0-1367) pg/mL; p=0.005) and SP (vaccinated 0.07(0-603.7) pg/mL; unvaccinated -102.2 (0-910.3) pg/mL; p=0.02). No differences were observed in the males for E co or SP responses (Fig 4-7B). However, IL-10 levels in males were significantly lower in the vaccinated compared to unvaccinated group after CLO-75 stimulation (vaccinated 23(0-232) pg/mL; unvaccinated 159(0-764) pg/mL; p= 0.04; Fig. 4-7D). This difference was seen in females in response to all TLR agonists: vaccinated group: LPS, 14 pg/mL (0-951); HKLM, 7(0-843) and CLO-75, 5(0-855)pg/mL compared to unvaccinated: LPS, 287(0-660) p=0.03), HKLM , 234(0-665) p=0.03) and CLO-75 (240(0-708); p=0.04; Fig. 4-7C).

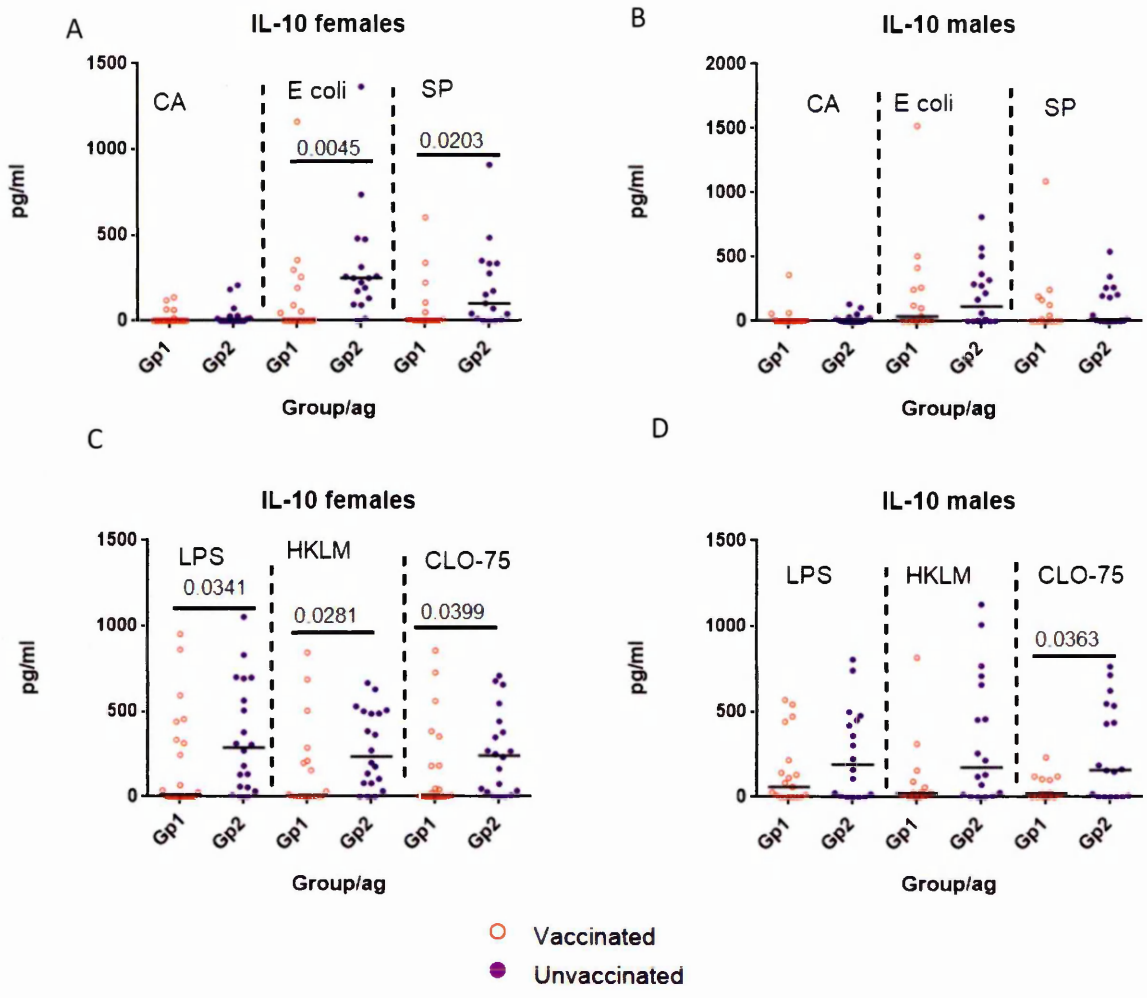


Figure 4-7: Differences between vaccinated (group 1) and unvaccinated (group 2) based on gender 1 week post vaccination: a) no differences were observed between males of the groups after unrelated pathogen stimulation;15 were from the vaccinated, 19 were from the unvaccinated ; b) vaccinated females had lower IL-10 levels after unrelated pathogen stimulation;18 subjects from the vaccinated group and 17 from the unvaccinated group; c) vaccinated males had lower IL-10 levels to TLR7/8 agonist only; 20 from the vaccinated and 18 from the unvaccinated group; d) vaccinated females had lower IL-10 levels to TLR 2, 4 and 7/8 agonists compared to their unvaccinated counterparts; 22 from the vaccinated group and 20 from the unvaccinated group.

4.4 Discussion

BCG is given together with OPV as part of the EPI vaccine schedule to Gambian children as recommended by WHO. Despite evidence that BCG might have heterologous benefits as well as protection against childhood TB, little is known about the immunological mechanisms.

It has been shown that neonates have an impaired Th1 response (Angelone et al., 2006; Philbin & Levy, 2009; Prabhudas et al., 2011; Cuenca et al., 2013). In this regard, the BCG-unvaccinated infants in our study had increasing Th2 cytokine levels over time whereas the vaccinated infants had no increase, suggesting a possible bias towards Th2 levels in the unvaccinated group. A decrease in plasma IFN-γ levels one-week post vaccination goes against the *modus operandi* of BCG vaccination in inducing IFN-γ (Marchant et al., 1999; Vekemans et al., 2001; Ota et al., 2002; Hanekom, 2005) however, these previous studies measured cytokine levels in supernatant after antigen stimulation as opposed to the unstimulated cytokine levels . The increase in IL-10 especially at 12 weeks post-vaccination in the unvaccinated and not in the BCG-vaccinated group could be due to the effects of an innate Th2-type bias caused by hepatitis B and DTP vaccines (received by both groups of infants) which is opposed and balanced by the prior Th1 enhancing BCG in the vaccinated group (Ota et al., 2002; reviewed in Marchant & Goldman, 2005).

Clearly the underlying cytokine milieu in the different vaccine study subjects will influence the antigen-specific responses we observed, however no difference in response to the positive control stimulus (PMA/Ionomycin) was observed between the groups suggesting that both groups of infants were equally able to respond to mitogenic stimulation. This shows that differences in the antigen-induced responses were probably due to vaccine-related effects.

In vitro stimulation with the BCG recall antigen PPD induced Th1 type responses, which were comparable between the groups suggesting no influence of BCG vaccination on the skewing of vaccine-specific immunity, which is in contrast to a number of studies from The Gambia (Ota et al., 2002; Burl et al., 2010) and elsewhere. The low responses following PPD stimulation might be due to the fact that we did an overnight stimulation which would mostly be measuring innate responses and the use of cryopreserved PBMCs as neonate PBMCs tend to be highly attenuated when cryopreserved, Burl and colleagues measured levels after 5-days culture in whole blood and had a much more robust response (Burl et al., 2010). Despite this, fold changes in Th1 cytokines from pre-vaccination levels were much higher in the vaccinated than the unvaccinated subjects whilst the unvaccinated had higher IL-10 fold changes. These results further support a skewing of infant immunity to Th2 that is altered with BCG vaccination, but perhaps due to our small sample size this was not significant.

The innate immune response is crucial for the activation of antigen presenting cells and induction of adaptive immunity. PRRs are integral to this with TLRs being among the most important activators of APCs. BCG has been shown to have both TLR2 and TLR4 agonist activity (Tsuji et al., 2000; Akira et al., 2001; Uehori et al., 2003). In regards to TLR agonist stimulation, we found no enhancement following BCG vaccination; the unvaccinated had higher cytokine levels than the vaccinated group at one week post vaccination. In contrast, a previous cross-sectional study in Gambian infants observed an increase in pro-inflammatory cytokine levels from birth when compared to later age-groups up to one year of age, whilst acute phase cytokines (IL-1β and IL-6) and IL-10 decreased over time, these changes were more prominent in the first week of life (Sarah Burl et al., 2011). This effect was not found in our study, as cytokine levels over time were quite similar regardless of vaccination status.

We measured the effect of BCG vaccination on cytokine production after heterologous pathogen stimulation but did not find any effect of BCG-vaccination, despite the fact that

BCG vaccination in mice protected against candidiasis and enhanced pro-inflammatory responses to heat killed pathogens in BCG vaccinated adults (Kleinnijenhuis et al., 2012). We found similar proinflammatory cytokine levels in both groups, and higher IL-10 production after *E coli* stimulation in the unvaccinated group at 7 and 18 weeks, suggesting that the BCG naive subjects had a skew towards an immunosuppressive response. One explanation might be the differences in vaccine strains used, since we used the Russian strain from India a much more attenuated strain while the Kleinnijenhuis study used BCG Copenhagen.

Several studies have found differences in inflammatory and antibody responses between males and females (Moxley et al., 2002; Casimir et al., 2010), however we did not find any differences in pro-inflammatory levels between the males and females after the various stimulations. Interestingly however, we found higher IL-10 (anti-inflammatory) levels in females in the unvaccinated compared to the vaccinated group in response to TLR agonists and whole killed pathogens. The observed lower IL-10 responses in the vaccinated females' points to an effect of BCG in modulating the production of this anti-inflammatory cytokine in response to non-vaccine related stimuli. Less IL-10 might lead to less immunoregulation and thus greater pathogen clearance. The lack of the same effect in males supports the long-asserted sex differences in heterologous effects of vaccines.

In conclusion, we found low plasma cytokine levels and little cytokine production to PPD and TT. Innate pro-inflammatory responses were more pronounced in the unvaccinated group, and the BCG vaccinated group had lower production of IL-10 to TLR agonists and unrelated pathogen stimulation when compared to their unvaccinated counterparts. This effect was more prominent in females. These findings therefore support the notion of sex-differential heterologous effects of BCG on innate immunity.

Chapter 5: Characterization of cytokine producing cells by flow cytometry

5.1 Introduction

5.1.1 The adaptive immune response to Bacillus Calmette Guerin (BCG)

Limitations in the development of the neonatal immune response places neonates at a much higher risk of infections compared to the adults, and vaccine responses may be poorer than in adults. Therefore vaccinations are essential in early-life to help boost the immune response to invading pathogens. BCG, used to protect infants against pulmonary TB, has been shown to induce two types of immune response: the classical antigen specific T cell response and an innate immune response based on functional reprogramming of the innate immune cells. Dubbed 'trained immunity', innate cells such as monocytes are provided with characteristics of cells of adaptive immunity i.e. innate cells exhibit memory characteristics (heightened response) to secondary infection, exerted both towards the same or different (cross-protection) pathogens (Netea et al., 2011; Kleinnijenhuis et al., 2012) . BCG vaccination induces different phenotypes of vaccine specific CD4 and CD8 T cells with the ability to produce both pro- and anti-inflammatory cytokines (Marchant et al. 1999; Hussey et al. 2002; Murray et al. 2006; Soares et al. 2008; Burl et al. 2010; Li et al. 2012). Long lived memory B cells have also been suggested to be induced after BCG vaccination in healthy adults (Sebina et al ., 2012). This array of responses induced after BCG vaccination contrasts with the theory that the infant immune response is "immature". Despite the induction of Th1 responses, it has been shown that IFN-γ production alone is not effective in imparting immunity needed for protection against TB (reviewed by Abebe, 2012). Since the measurement of a single cytokine at a time is believed to underestimate not only the complexity but magnitude of induced immunity, most recent studies carry out polyfunctional cytokine analysis as a better indication of mycobacterial induced immunity, however this is not correlated to protection (Kagina et al., 2010). This allows us to identify cells secreting more than one cytokine simultaneously. Vaccination at birth has been shown to elicit both CD4+ and CD8+ polyfunctional cells in 10 week old South African infants (Soares et al., 2008), whilst another study by the same group observed that CD4 T cells were more likely to be

polyfunctional in infants that had BCG at 10 weeks when compared to those that were vaccinated at birth (Kagina et al. 2009). A cross-sectional study of 9 month old Ugandan infants observed that infants vaccinated at birth had not only higher BCG-specific IFN-γ alone, but also higher frequencies of T cells co-expressing IFN-γ, IL-2 and TNFα when compared to infants vaccinated at 6 weeks of age (Lutwama et al., 2013).

In the previous chapter we found differential cytokine patterns following stimulation of BCG-vaccinated compared to unvaccinated infants with different antigens, including TLR agonists. However, we could not determine the source of the cytokines in these assays.. Thus we investigate the phenotype of cells producing cytokines after overnight stimulation with vaccine specific and non-related antigens using intracellular cytokine staining by flow cytometry in this chapter. No gender-based differences were analyzed in this chapter due to a disparity in numbers.

Hypothesis:

- Infants vaccinated with BCG at 6 weeks of age would have higher cytokine responses from different cell subtypes after mycobacterial and unrelated pathogens stimulation compared to BCG naïve infants
- Infants vaccinated with BCG at 6 weeks would have higher polyfunctional cytokine responses from different cell subsets after mycobacterial and unrelated pathogen stimulation compared to BCG naïve infants.

Aims:

- Investigate cell phenotypes producing cytokines in stimulated cryopreserved PBMCs from BCG vaccinated and naïve infants
- Investigate polyfunctionality of these cell phenotypes after stimulation with vaccine specific and non-specific antigens.

5.2 Methods

5.2.1 Study population and sample characteristics

142 pregnant mothers were sensitized about the study and 137 consented to take part in the study. 131 infants were bled at 6 weeks, of these 66 were randomized into group 1 (vaccinated) whilst the rest were randomized into group 2 (delayed/unvaccinated); 66 were females and 71 were males. 105 infants completed the study (53 in vaccinated group, 52 in the unvaccinated group). After cryopreservation of peripheral blood mononuclear cells (PBMCs), only samples with a viability of at least 60% were included due to a limitation in cell numbers as well only 41 subjects with cells for all three time points were included in the final analysis, 15 were in the vaccinated group(10 males, 5 females) whilst 26 were in the unvaccinated group (11 males,15 females) .

5.2.2 PBMC thawing, stimulation and staining

PBMCs were thawed at 37°C in a water bath (Section 2.4.3), resuspended in 10% Fetal Calf Serum (FCS) in RPMI plus a final concentration 25 units/mL of Benzonuclease (Sigma Aldrich. USA). Cells were washed twice with 10% FCS in RPMI, resuspended in 10% FCS in RMPI plus Benzonuclease and rested for 6 hours at 37°C 5% CO_2. 0.5 x 10^6 cells were stimulated per antigen: a negative control (medium only), a positive control (PMA/Ionomycin) PPD and TT (vaccine specific antigens), Esat-6/CFP-10 (*Mtb* specific antigen) and *Streptococcus pneumoniae*, *Candida albicans* and *E. coli* (Unrelated pathogens) as described in Chapter 2 (section 2.4.5.1 and 2.4.5.2). Cells were then incubated for 2 hours prior to the addition of Brefeldin A (10µg/mL), after which an overnight incubation (16-18 hours) at 37°C was carried out. After overnight stimulation, cells were washed and stained with Live/Dead Aqua yellow, incubated for 10 mins and surface markers (CD3, CD8) added. Cells were then incubated at room temperature for 30 minutes in the dark after which they were fixed, permeabilised and stained for Intracellular cytokines (IFNγ, TNFα, IL-2, IL-10 and IL-17). After 30 minutes incubation in the dark, the cells were washed, resuspended in FACS buffer and acquired using a 9-colour Cyan ADP (Beckman Coulter, USA).

5.2.3 Statistical analysis

ICS data were analyzed using FlowJo v10.1 (Treestar, USA). Frequencies of cytokine expression in the negative control samples were subtracted from antigen specific responses. Boolean gating was carried for each pro-inflammatory cytokine to allow for combinatorial analysis. A total of fifteen distinct cytokine subsets were generated. Statistical analysis was done using GraphPad Prism V6 (GraphPad Software, Inc., La Jolla, USA). Mann-Whitney U test was used to compare between two groups and Kruskal-Wallis test was used to compare between three groups, a q-value of 0.04 was considered significant using a false discovery rate of 5%. Polyfunctional analysis was performed using the SPICE software (this software is provided free of charge by the National Institute of Allergy and Infectious Disease), using a false discovery rate of 5% a q-value of 0.035 was considered significant.

5.3 Results

5.3.1 Phenotypic characterization of cytokine-producing cells by flow cytometry

Different cell phenotypes have been shown to be responsible for the induction of different cytokines during vaccine induced and natural immunity. We have previously shown patterns and amount of cytokine production to vaccine-specific and non-specific antigens (Chapter 4). The aim of this chapter was to determine the cell types producing these cytokines using flow cytometry. We used surface staining for T cell markers and intracellular cytokine analysis for proinflammatory cytokines (IFN-γ, TNF-α, IL-2 and IL-17) and anti-inflammatory (IL-10). Because cryopreserved cells were used, cells with viabilities less than 60% were excluded from analysis. 41 subjects had flow cytometry data included in the final analyses: 15 BCG-vaccinated and 26 unvaccinated infants. We first gated on the lymphocyte population (Fig. 5-1A), excluded doublets (Fig. 5-1B) and gated out the dead cells using live–dead viability stain (Fig. 5-1C). We were then able to gate on CD3+ and CD3- neg cells (used for innate cell analysis) (Fig. 5-1D). Within the CD3+ population we then gated on CD4+ and CD8+ T cells (Fig. 5-1E). Within each of these populations, all cytokines were then analyzed using Boolean gating (Fig. 5-1F).

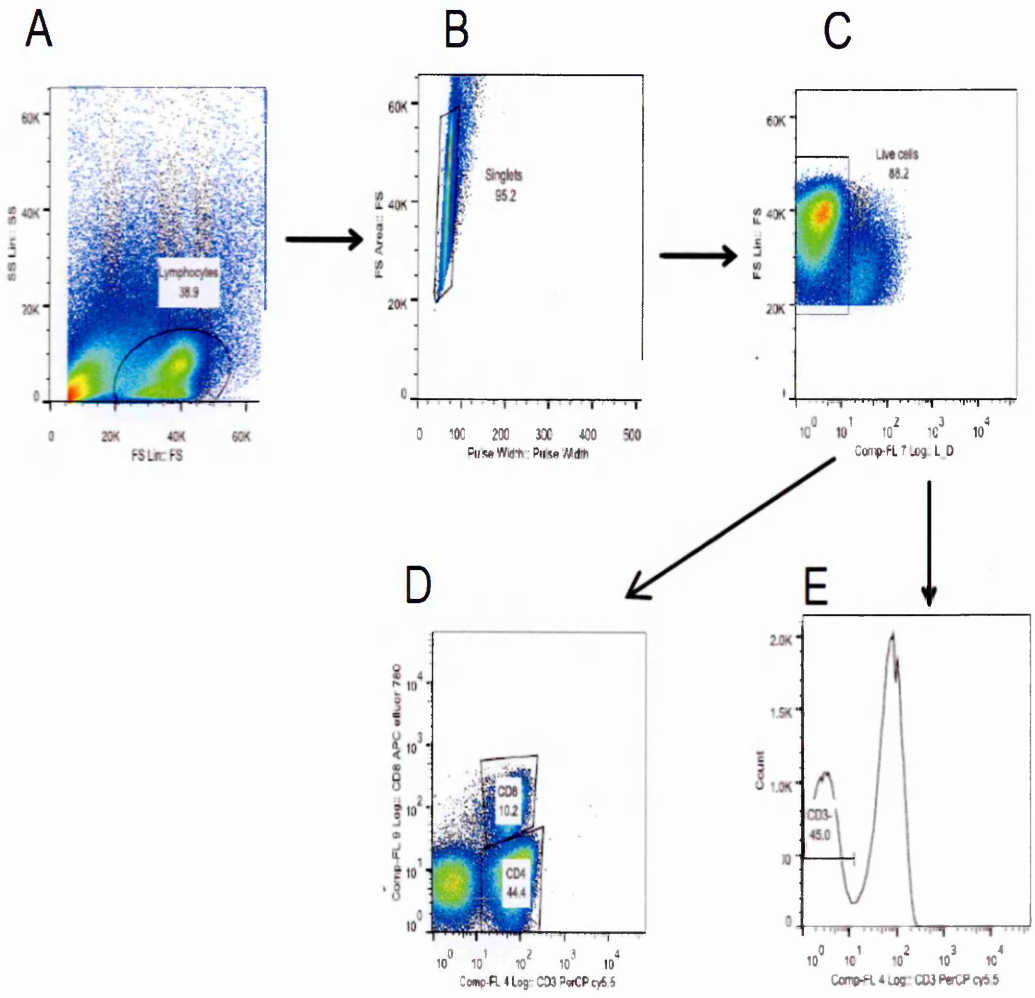


Figure 5-1: The gating strategy used in identifying cytokine producing CD4+, CD8+ and CD3- cell subsets. Lymphocytes were gated on first (A) before Doublet discrimination (B); viable cells were gated on by gating out live-dead positive cells(C). Non- T cells were gated on by gating by gating on CD3-neg cells (D), whilst CD8 and CD4+ cells were gated on the CD3+ population (E).Plots representative of all samples.

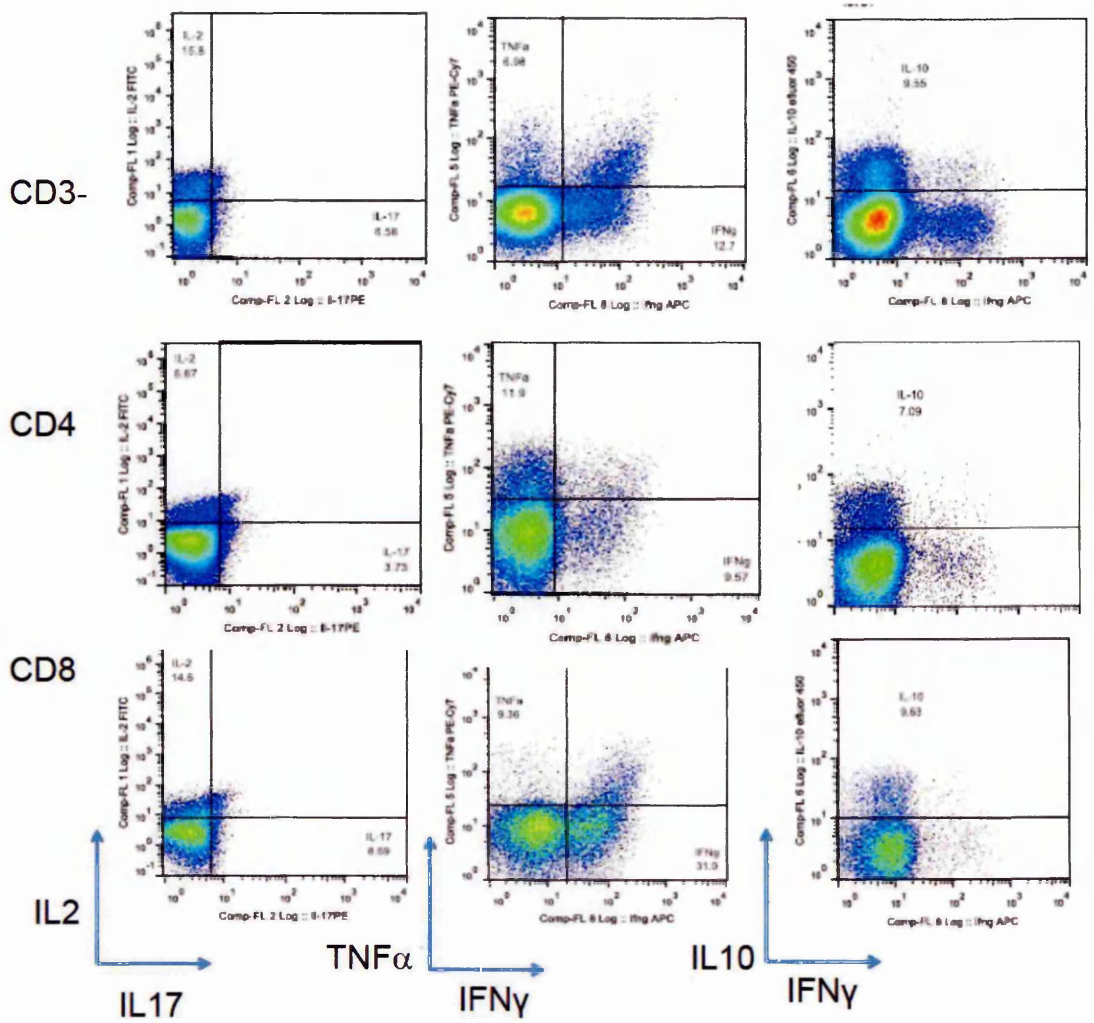


Figure 5-2: ICS plots of cytokine production in response to PMA /Ionomycin stimulation. The top plots show non-T cells (CD3- neg), middle plots CD4 + T cells and the bottom plots show CD8+ T cell cytokine productions. Plots from left to right: IL-2 Vs IL-17, TNFα Vs IFNγ and IL-10 Vs IFNγ. Plots are representative of all samples.

5.3.2 Responses to a polyclonal stimulator (PMA-ionomycin (PMA-I))

Following overnight stimulation with PMA-I comparable proportions of pro-inflammatory cytokine producing cells were observed from all cell types analyzed (CD4+, CD8+ and non-T cells) and this was alike in both groups at all time-points, indicating similar capacity to respond regardless of vaccination status. This is also what was found with the multiplex cytokine analysis in Chapter 4. No difference in single-positive IL-10 or IL-17 producing T cells was seen between groups at any time-point. However, the proportion of CD3- neg IL10+ cells increased from 1 week post-vaccination to 12 weeks post-vaccination in the vaccinated group, (median (IQR): 1 week post-vaccination ,0.06 (0-1.50); 12 weeks post-vaccination, 0.20(0.03-1.60); p=0.0040) and levels were higher in the vaccinated than the unvaccinated infants at 12 weeks post-vaccination (median (IQR): unvaccinated, 0.02(0-0.70); p=0.0022) (FIG 5-3B). The same trend was observed for IL-17 production from non-T cells (CD3-neg) increasing from 1 week post-vaccination to 12 weeks post-vaccination in the vaccinated group but this was not significant (median (IQR): 1 week post-vaccination, 0.06(0-2.50); 12 weeks post-vaccination, 0.20(0.01-2.70); p= 0.0300) and the vaccinated infants had more CD3-neg cells producing IL-17 at 12 week post-vaccination compared to the unvaccinated group (median (IQR): unvaccinated, 0.06 (0-1.5); p=0.008) (Fig 5-3A).

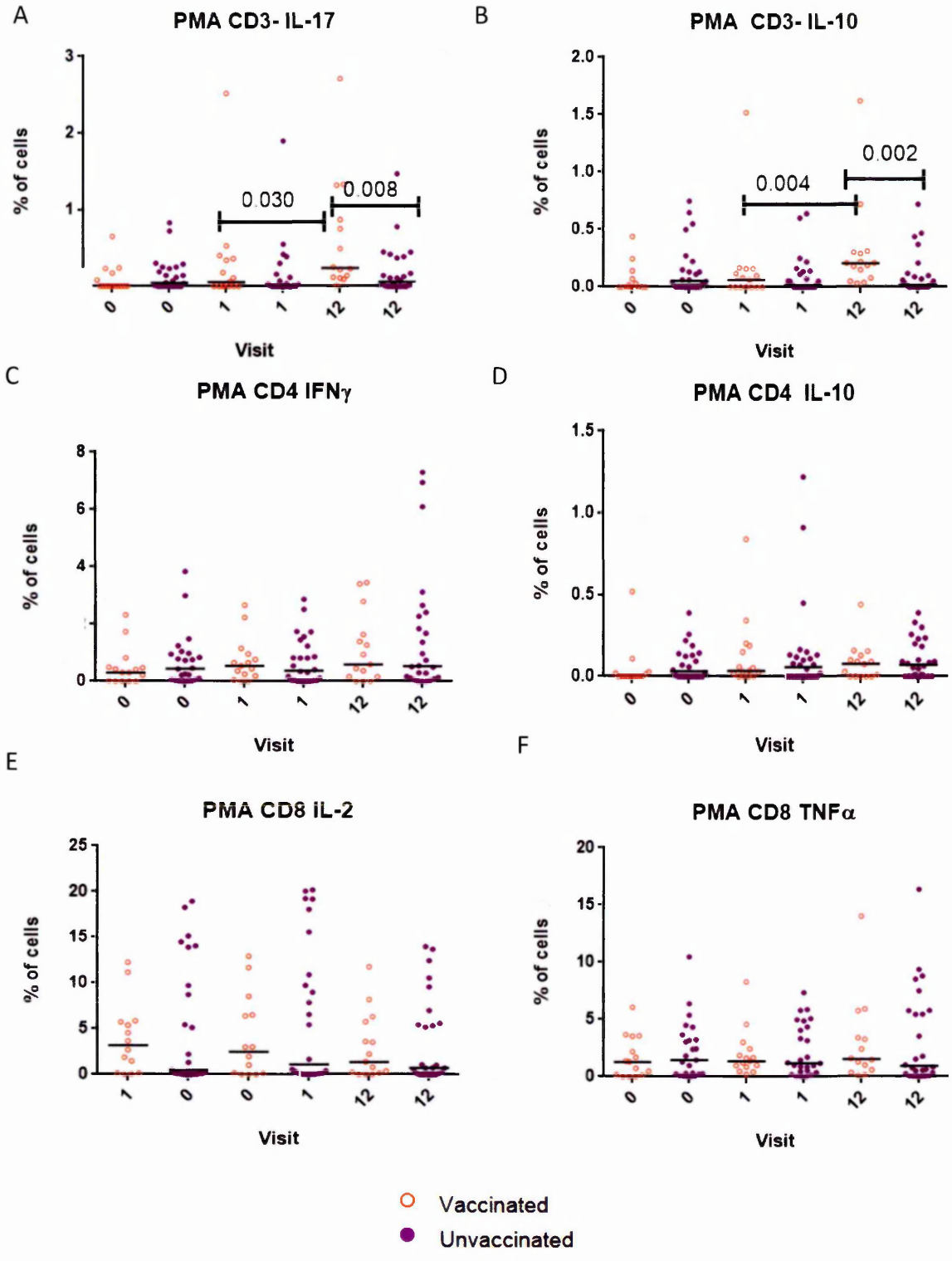


Figure 5-3: Cytokine production from the different cell phenotypes after PMA/Ionomycin stimulation (A&B) the vaccinated group had increased CD3-neg expressing IL-10 from 1 to 12, and higher than the naive group at 2 and this was observed for IL-17 response as well. (C-F) No differences were observed from CD4 and CD8 T cell cytokine production between the groups from any of the groups. Open red circles represent the vaccinated, closed purple circle represent the unvaccinated; 0- 6 weeks/baseline;1-7 weeks/ 1-week post vaccination; 12- 18 weeks/ 12 weeks post-vaccination. Data is representative of 26 unvaccinated subjects and 15 vaccinated subjects at all time points.

5.3.3 Vaccine specific antigens elicit differential cell-specific cytokine production

Vaccine specific cytokine production was measured from the different cell phenotypes following stimulation with PPD as a marker of BCG vaccine induced responses and TT as the tetanus specific component from the DTP vaccine. We found surprisingly low cytokine production after PPD stimulation. However, while no difference in response from CD4+ T cells was observed (FIG 5-4 A & B) and we observed higher levels of CD8+ IFNγ+ T cells, 1 week post-vaccination in the vaccinated compared to the unvaccinated ($p=0.04$ (border-line significance)) (FIG 5-4 C).1 week post-vaccination CD3- neg (non-T) cell production of IL-17 increased a in the vaccinated group but decreased in the unvaccinated group, this was not significant (FIG 5-4E). A highly significant increase in TNF-α production from the CD3- neg cells at 1 week post-vaccination was observed in the vaccinated group compared to pre-vaccination levels and this was also significantly higher than the unvaccinated group ($p=0.0002$ and $p<0.0001$, respectively) (FIG 5-4F). However, these levels were not maintained with a significant decrease observed in the vaccinated group by 12 weeks post-vaccination ($p<0.0001$) (FIG 5-4F).

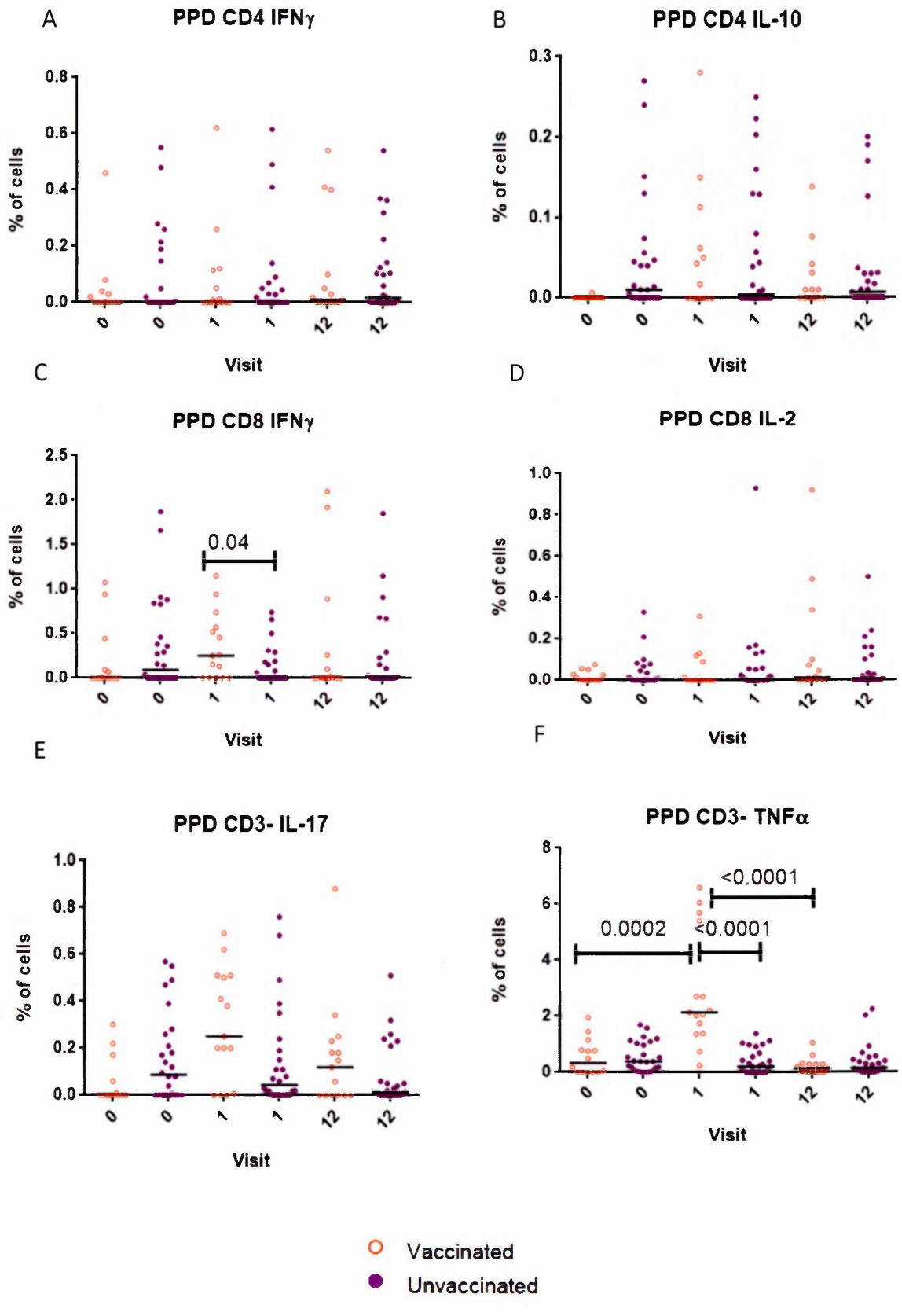


Figure 5-4: Vaccine specific PPD responses from the different cell phenotypes. Red open circles represent the vaccinated , purple closed circles represent the unvaccinated; 0-baseline/ 6 weeks, 1-1 week post-vaccination/7 weeks, 12-12weeks post-vaccination/18 weeks. Data were analyzed using Mann Whitney U test, significant differences are indicated. Data is representative of 15 vaccinated subjects and 26 unvaccinated subjects

5.3.4 Cytokine responses following ESAT-6/CFP-10 stimulation

In order to measure infants' sensitization to tuberculosis we measured cytokine responses using the *Mycobacterium tuberculosis (Mtb)* specific antigen ESAT-6/CFP-10, which is not present in BCG or environmental mycobacteria. Whilst very little cytokine reactivity was observed from CD4 and CD3- neg cells (Fig 5-5A-B, 5-5E-F), the vaccinated group had decreased CD8+IFN-γ production from 1 week post-vaccination to 12 weeks post-vaccination ($p=0.002$); Fig 5-5C).

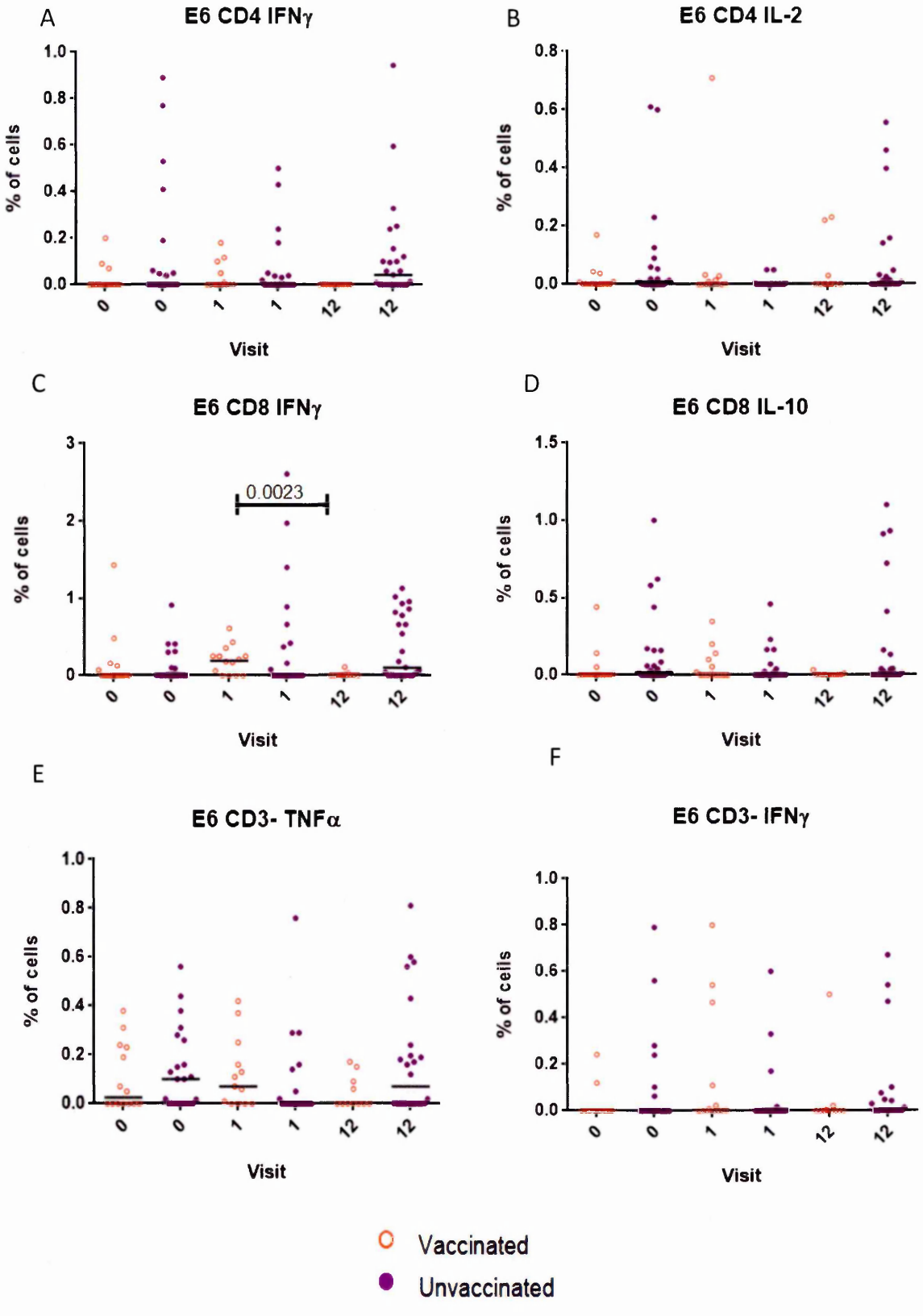


Figure 5-5: Cytokine production from cell phenotypes after Esat-6/CFP-10 stimulation, red open circles represent the vaccinated group, purple closed circles represent the unvaccinated group; 0- 6 weeks/ pre-vaccination, 1-7 weeks/1 week post-vaccination,12-18 weeks/12 weeks post-vaccination. Data was analyzed using Mann Whitney U test, significant differences are indicated. Data represents 18 vaccinated subjects and 24 unvaccinated subjects.

5.3.5 Cytokine production after non-specific pathogen stimulation

In response to fungal (*C. albicans* (CA)) antigen stimulation little cytokine production was observed from CD4 and CD3-neg cells. It is important to note that all data presented is following background subtraction so all results are specific for the antigen of interest, thus these findings indicate a definite responsiveness of cells in the vaccinated group to both vaccine-specific and non-specific antigens. An increase in CA-specific CD8+ IFN-γ+ cells was seen in the vaccinated group at 1 week post vaccination compared to baseline ($p=0.02$) and this decreased at 12 week post-vaccination ($p=0.04$). Despite the fact that IL-10 levels from the previous chapter were higher in the unvaccinated than the vaccinated in response to all three pathogens, we did not find any differences in IL-10 levels to any of the pathogens from CD4, CD8 and CD3-neg cells by flow cytometry (Fig 5-6).

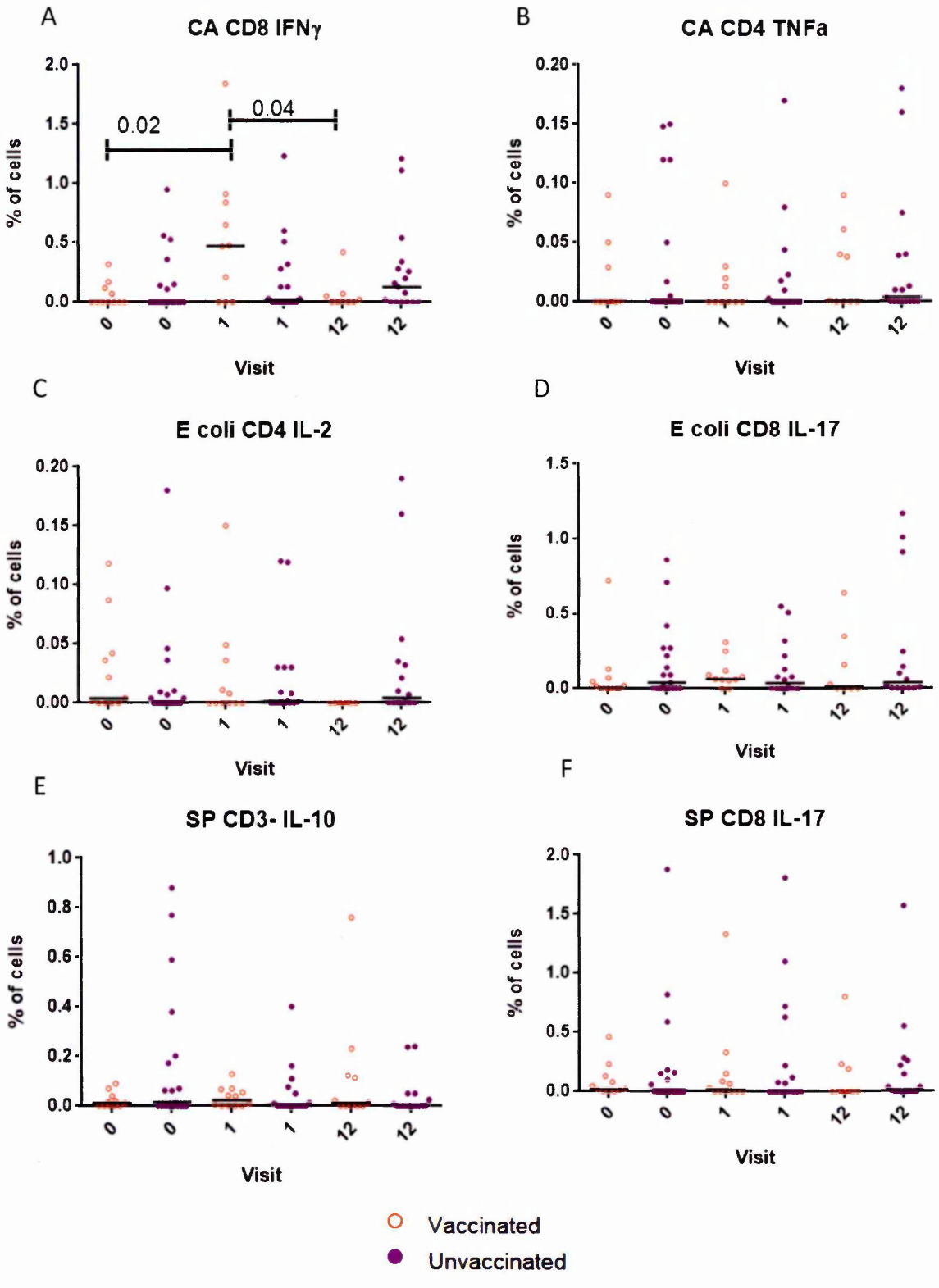


Figure 5-6: Cytokine production after unrelated pathogens stimulation at 0(6 weeks), 1 (7 weeks/1 week-post vaccination) and 12 (18 weeks/12 weeks-post vaccination). Red open circles represent the vaccinated whilst the purple closed circles represent the unvaccinated group. Data represents 9 vaccinated subjects and 21 unvaccinated subjects.

5.3.6 Polyfunctional cytokine responses

We next measured qualitative polyfunctional cytokine responses from CD4 and CD8 T cells. Boolean gating on FlowJo was done for different combinations of IFN-γ, TNF-α, IL-2 and IL-17 positive cells with a total of 15 possible combinations,1 possible combination for all four cytokines (IFNγ+IL-2+TNF-α+IL-17+); 4 possible combinations for simultaneous production of three cytokines (IFNγ+IL-2+TNF-α+IL-17-), (IFNγ+IL-2+TNF-α-IL-17+), (IFNγ+IL-2-TNF-α+IL-17+), (IFNγ-IL-2+TNF-α+IL-17+); 6 possible combinations for simultaneous production of two cytokines only (IFNγ+IL-2+TNF-α-IL-17-), (IFNγ+IL-2-TNF-α+IL-17-), (IFNγ+IL-2-TNF-α-IL-17+), (IFNγ-IL-2+TNF-α+IL-17-), (IFNγ-IL-2-TNF-α+IL-17+),(IFNγ-IL-2+TNF-α-IL-17+); and 4 possible single cytokine only production (IFNγ+IL-2-TNF-α-IL-17-), (IFNγ-IL-2+TNF-α-IL-17-), (IFNγ-IL-2-TNF-α+IL-17-),(IFNγ-IL-2-TNF-α-IL-17+) . A q-value of 0.035 was considered significant.

5.3.6.1 Polyfunctional T cell analysis

In response to PMA/Ionomycin, no differences were found between the groups for both CD4 and CD8 producing T cells (Fig 5-7).

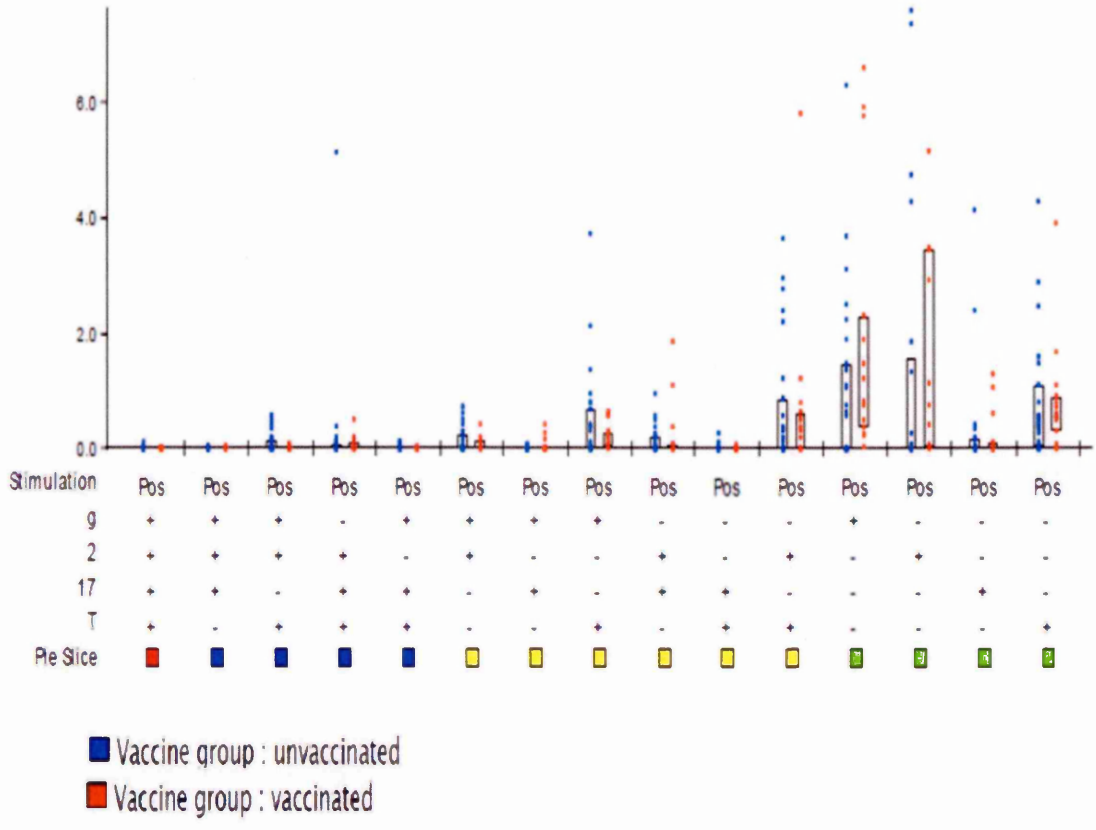
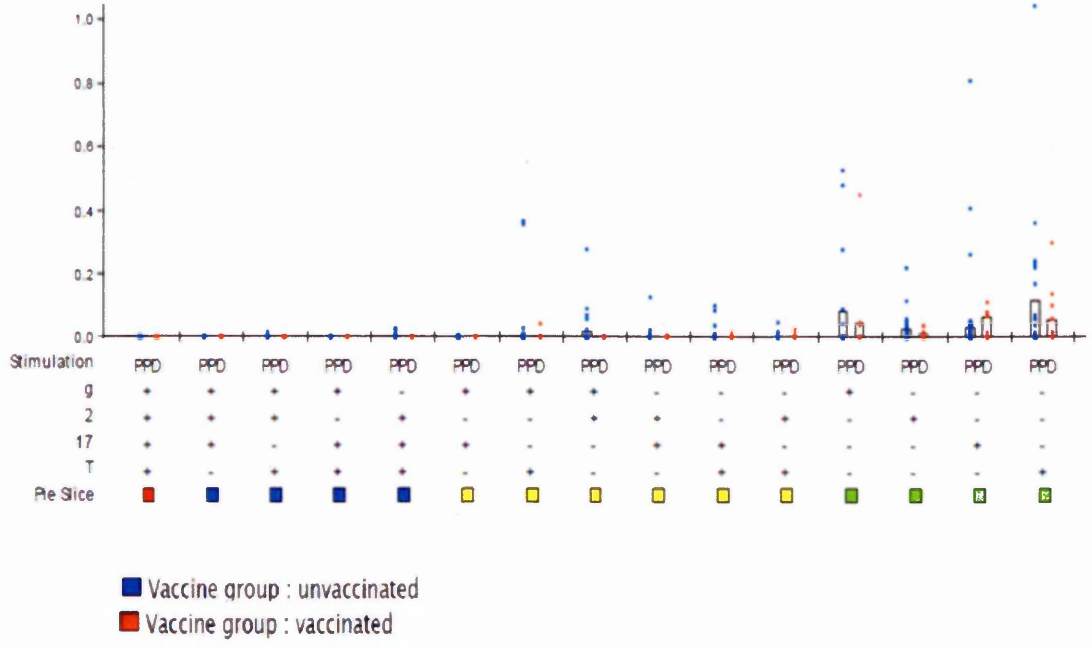


Figure 5-7: Combinations of cytokine production after PMA/Ionomycin stimulation from CD8 T cells at 1 week post-vaccination. No differences were observed between groups at any time point after polyfunctional cytokine responses analysis. Y axis represents absolute number of cells producing polyfunctional cytokines. Data presented represents 26 unvaccinated subjects and 15 vaccinated subjects.

In response to PPD the unvaccinated group had higher proportions of CD4+ T cells producing any two cytokines at baseline compared to the vaccinated group (pie graphs)(Fig 5-8b). No differences were observed between the groups however after individual combination analysis at this time point (Fig. 5-8a). Additionally, the vaccinated infants had lower single cytokine production but this was not significant (Fig 5-8a).

A.



B.

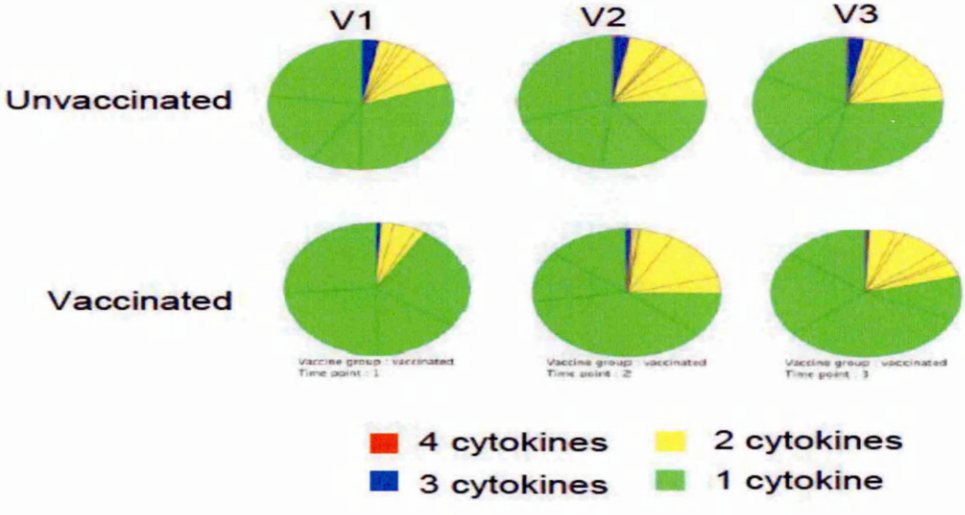
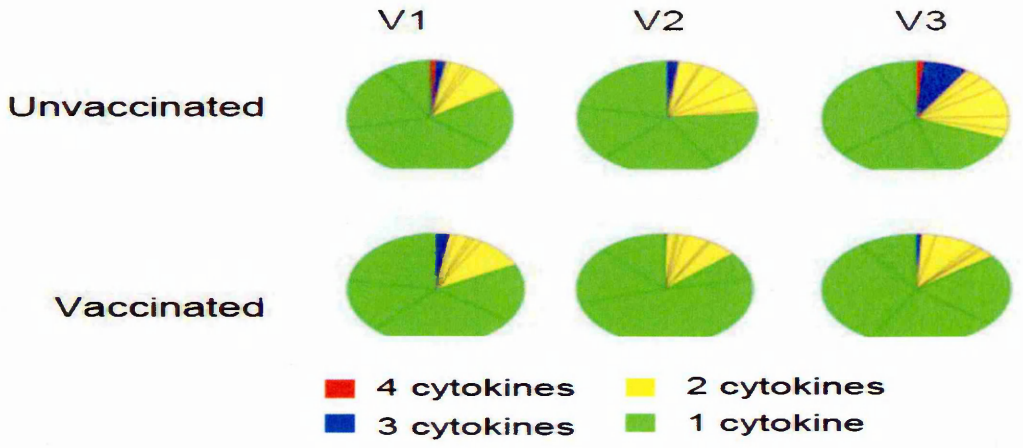


Figure 5-8: Proportions and cytokine production combinations from CD4 T cells after PPD stimulation a) cytokine production and the different possible combinations at 6 weeks; absolute percentage of cells are on the y-axis and cytokine combinations on x-axis, b) different proportions of cytokine production over time in the unvaccinated (top row) and the vaccinated (bottom row).Data represents 15 vaccinated and 26 unvaccinated subjects

CD8 T cells from the vaccinated group had lower ability to produce multiple cytokines simultaneously, though this observation was not significant (Fig 5-9). No differences were observed in terms of cytokine combinations between the groups after multiple comparisons.

A.



B.

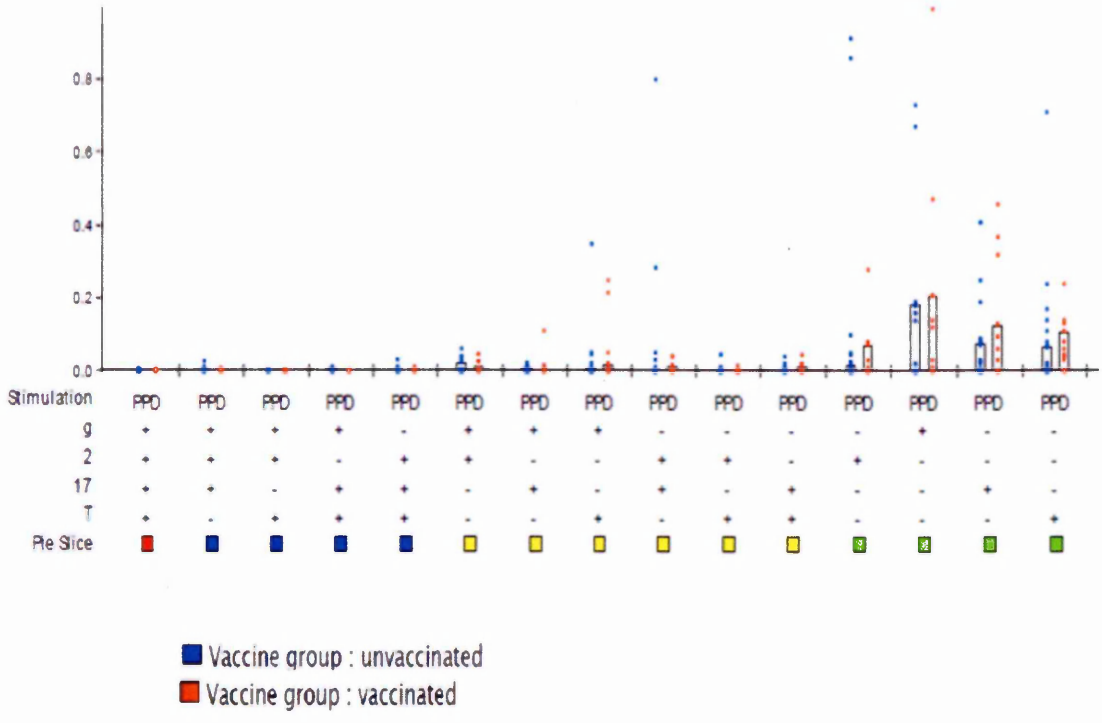


Figure 5-9: Proportions and combinations of cytokine production from CD8 T cells after PPD stimulation, a) proportions of different cytokine combinations over time, unvaccinated are in the top row, bottom row, vaccinated b) the possible combinations at 7 weeks from the vaccinated and unvaccinated, p-values calculated on SPICE using the Wilcoxon rank sum test. Y-axis is absolute number of cells producing polyfunctional cytokines; data represents 15 vaccinated and 26 unvaccinated subjects.

Equivalent proportions of multiple and single cytokine production was observed between the groups at all time points, no differences were observed in the simultaneous production of multiple cytokines (Figs. 5-10a & b).

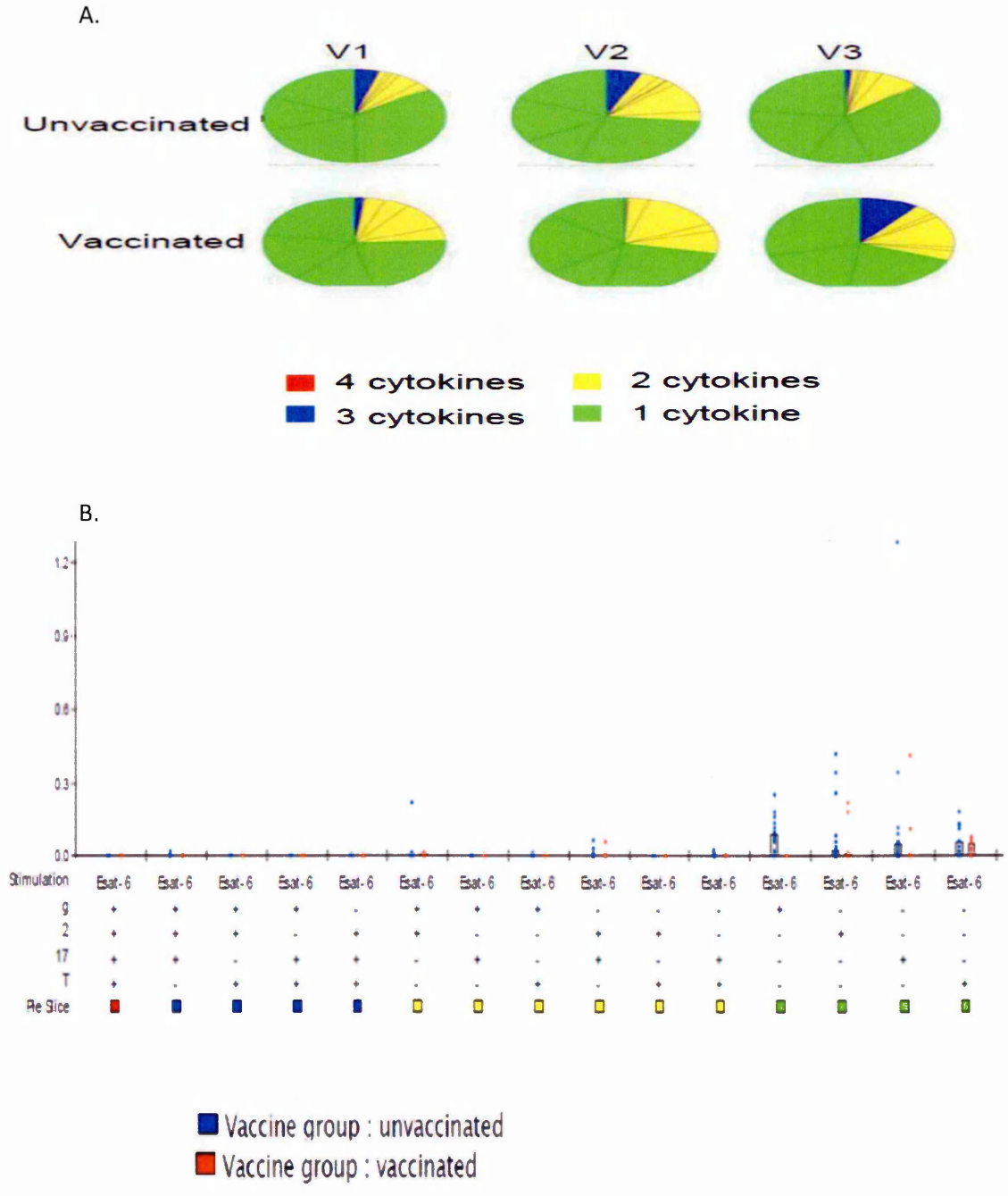


Figure 5-10: Proportions and cytokine production combinations from CD4 T cells after Esat-6/CFP-10stimulation a) different proportions of cytokine production over time in the unvaccinated (top row) and the vaccinated (bottom row), b) cytokine production and the different possible combinations at 12 weeks post-vaccination; absolute percentage of cells are on the y-axis and cytokine combinations on x-axis. P-values calculated on SPICE using the Wilcoxon rank sum test. Data represents 26 unvaccinated infants and 15 vaccinated infants

CD8+ T cell production of polyfunctional cytokines was more apparent in the unvaccinated than the vaccinated infants at baseline (not significant), similar proportions were observed at 1 week post-vaccination and 12 week post-vaccination. After combinatorial analysis no differences were observed between the groups at all time points (data not shown).

Polyfunctional T cell responses after unrelated pathogen stimulation varied. Similar proportions of CD4 T cells simultaneously producing 3 cytokines per cell were observed after *Candida albicans* at all time points between the two groups. Following *E. coli* stimulation similar proportions of single and multiple cytokine producers were observed between the groups. Simultaneous production of 4 cytokines was observed in the vaccinated after *S. pneumoniae* stimulation at 1 week post-vaccination; equivalent proportions of other possible combinations were observed. No differences were observed between the groups from CD8+ T cells in terms of both polyfunctionality and single cytokine production in response to unrelated pathogens (Fig 5-11).

A

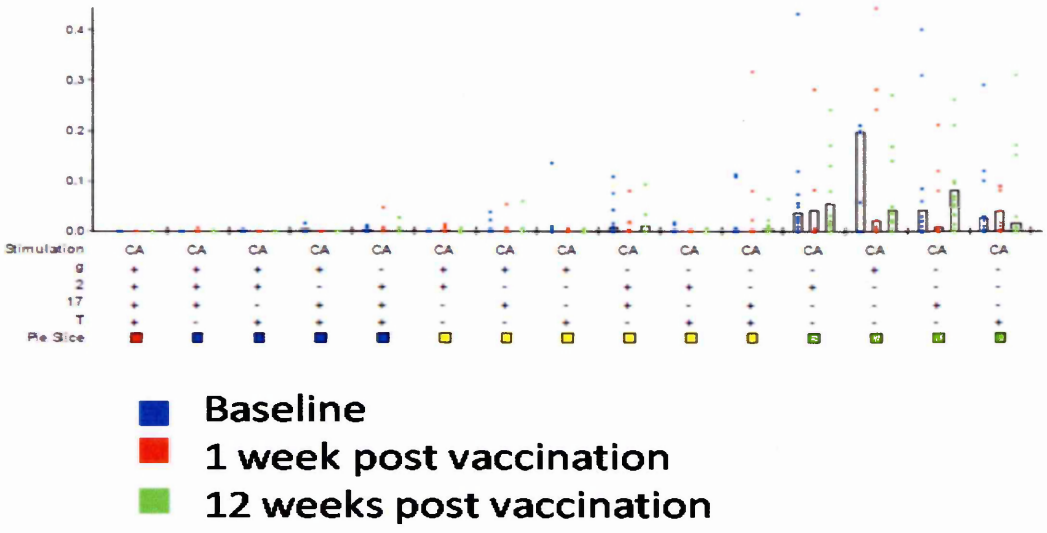


Figure 5-11: cytokine production and the different possible combinations after unrelated pathogen stimulations; a) CA responses in the unvaccinated group over time b) E coli levels at 12 weeks post vaccination; absolute percentage of cells are on the y-axis and cytokine combinations on x-axis. P-values calculated on SPICE using the Wilcoxon rank sum test

5.4 Discussion

In this chapter, we compared cellular responses between the vaccinated and unvaccinated groups. CD4+ T cells are the primary producers of IFN-γ and other pro-inflammatory cytokines after BCG vaccination in S. Africa and Uganda (Vekemans et al. 2001; Soares et al. 2008; Lutwama et al. 2013) but there are little data on the cellular source of cytokines in Gambian infants, nor is there information on what delaying BCG vaccine means to overall immunity (both innate and adaptive). We have shown in Chapter 4 that plasma cytokines (i.e. basal cytokine levels) are different between the vaccinated and unvaccinated infants suggesting this will affect the antigen-specific responses both at the global and single-cell level.

In this chapter, Th1, Th2 and Th17 cytokine profiles were examined from T and non-T cells both early and late post-vaccination compared to non-vaccinated infants. Interestingly, there was no difference in CD4 T cell Th1 cytokine production at any time point after BCG vaccination, in contrast to previous studies in S. Africa. However, there was a significantly higher proportion of IFN-γ producing CD8+ T cells in vaccinated infants at 1 week post-vaccination. In addition, an increase in non-T cell production of TNF-α production 1 week after vaccination was evident in the vaccinated group and supports a role for the innate immune response early post-vaccination. We also measured Th17 producing cells, which were recently implicated in the protective immune response after BCG vaccination (Scriba et al., 2008; Li et al., 2012), although another paper showed no IL-17 induction after BCG vaccination in UK adolescents (Smith et al., 2010). In Gambian infants, quite high levels of IL-17 production were seen following antigen stimulation but no difference was seen between groups and responses were similar following all stimulation with antigens (both vaccine-related and unrelated) indicating this is not due to BCG vaccination. Smith *et al.* attributed their lack of an IL-17 subset in their studies to the use of PBMCs, as Scriba *et al.* used whole blood assays. In this study we used cryopreserved PBMCs to measure our cytokine responses, and we only found responses from non-T cells. Smith *et al.* measured IL-17 from T cells and NK cells. It is possible that

other non-T cells (mainly innate cells) are responsible for IL-17 production and not NK cells.

To analyze specific Mtb responses, we used Esat-6/CFP-10 (EC) overlapping peptides. Esat-6/CFP-10 is a part of the region of differentiation 1 (RD1) of the Mtb genome that is deleted in BCG and in environmental mycobacteria and therefore gives an indication of Mtb sensitization in our infants. CD4 IFNγ levels after Esat-6/CFP-10 simulation is indicative of TB infection via ELISpots (Adekambi et al., 2012).This was particularly important for the non-vaccinated group, since any evidence of infection would have warranted immediate investigation for TB infection. Low levels of cytokine production were observed after Esat-6/CFP-10 stimulation suggesting that none of the infants in our study had been exposed to TB. Indeed, a previous study in the same setting observed very low IFN-γ levels to Esat-6/CFP-10 in 18 month old infants using IFN-γ-ELISpot assay, corroborating our findings of low TB exposure (Odutola et al., 2012).

Our low response to PPD even in the vaccinated group was unexpected. It has been observed that prior environmental exposure might lead to an attenuated effect on the immune response after BCG vaccination (Black et al., 2001; Ota et al., 2002; Burl et al., 2010), however other studies showed no effect of exposure on attenuation of vaccine specific responses after delayed vaccination (Marchant et al., 1999; Kagina et al., 2009). Furthermore Kagina and colleagues describe the induction of a better immune response and protection a year after BCG vaccination in infants that were vaccinated at 10 weeks compared to those vaccinated at birth (Kagina et al., 2009), whilst another study in Ugandan infants observed higher cytokine levels in infants vaccinated at birth than those vaccinated at 6 weeks (Lutwama et al., 2013).In addition we measured BCG responses 1 week post-vaccination mainly because we wanted to measure innate responses and another arm of the study not discussed here looking at gene transcripts, a recent study observed that BCG vaccine responses peak after 10 weeks of vaccination gradually waning till 40 weeks and returning to almost baseline levels (3 weeks post-vaccination) at 1 year of age in BCG birth vaccinated infants (Soares et al., 2013). Thus, another reason

for the low responses we see especially at one week post vaccination might be due to the timing of our bleeds.

BCG has also been associated with protection against sepsis and bacterial infections in neonates and infants in some studies (Aaby et al., 2011). It has recently been observed that BCG also protects against *Candida albicans*, *Staphylococcus aureus* and *Escherichia coli* infection by the Netea group (Kleinnijenhuis et al., 2012). We therefore measured cytokine responses to CA, SP, and E coli (obtained from the Netea group) using similar pathogen concentrations as the Netea group. Overall, we did not find an effect of BCG on the immune response against unrelated pathogens.

Polyfunctional T cells in lungs of mice have been shown to correlate with protection against TB (Forbes et al., 2008), and this was found to be dose dependent (Aagaard et al., 2009). Since then human studies have been exploring if polyfunctionality could be used as a correlate of protection especially in clinical trials. However, a recent study suggested that polyfunctionality might not be a correlate, as development of TB did not depend on the level of polyfunctionality from CD4+ T cells in S. African infants (Kagina et al., 2010). We did not observe any specific differences in polyfunctionality between the vaccinated and unvaccinated groups; indeed the unvaccinated generally had higher (though not significant) proportions of polyfunctionality, corroborating the above study that polyfunctionality might not necessarily be linked to protection, as the unvaccinated are yet to receive the vaccine and thus it is not expected that they would be protected against TB. It would be interesting to have a longitudinal study to further clarify the results and to determine TB incidence in this setting.

Chapter 6: Effect of delayed BCG vaccination on antibody levels to other Expanded Programme of Immunization vaccines and the tuberculin skin test

6.1 Introduction

The Expanded Program on Immunization (EPI) vaccinations play a major role in preventive medicine and public health (Kollmann, Levy, & Hanekom, 2013). The EPI schedule is comprised of several vaccines against different infectious diseases; either killed or live attenuated vaccines as described in Chapters 1 and 2. It has been suggested that each group of these vaccines have either a non-vaccine related beneficial (live vaccines) or detrimental (killed vaccines) effect depending on the sex of the child, the timing of vaccination and subsequent interactions with other vaccines(Aaby et al., 2012). The vaccine that has been suggested to have the highest beneficial effect on the protective antibody levels of other EPI vaccines is BCG (Ota et al., 2002; Ritz et al., 2013), which is generally given at birth but might sometimes be delayed due to personal (i.e. inaccessibility of health centres), national (i.e. war) and/or economic circumstances. Indeed, it has recently been accepted by the WHO that BCG vaccination at birth has a heterologous effect in reduction of all cause mortality (WHO, 2014).

In this chapter the effect of BCG given at 6 or 18 weeks on antibody levels to other EPI vaccines given several weeks later was determined. Additionally, tuberculin skin test (TST) reactivity was assessed to determine the influence of delayed vaccination on mycobacterial immunogenicity.

6.1.1 Efficacy and interaction between vaccines in the EPI schedule and sex differences after vaccination

Most serious infections occur in the first year of life when the immune system is still developing; hence a major goal of human medicine is efficacious vaccination within this first year. However, most vaccines were designed empirically without knowledge of the potential interaction with other vaccines in the EPI schedule. Recent studies have begun

to assess these interactions with both positive and negative influences uncovered, which appear to be sex-dependent (Flanagan et al., 2013). In the present study we were interested in the interaction of BCG on the common vaccines given in The Gambia, namely polio, pertussis, tetanus, diphtheria and hepatitis B vaccines. In The Gambia, pertussis is given in combination with diphtheria (D) and tetanus (T) vaccines as DT-whole cell Pertussis (DTwP) vaccine at 2, 3 and 4 months (see Table 1, chapter 1). Polio and Hepatitis B vaccines are given at birth and together with the DTwP vaccine as part of the pentavalent vaccine. Combining multiple antigens in a single injection is not only convenient but leads to better compliance and, importantly, it has been shown that the above mentioned antigens combined with hepatitis B do not impact on each other's immunogenicity (Prymula & Plisek, 2008). However, data from a trial of a novel TB vaccine in The Gambia (MVA85A) showed it had detrimental effects on other EPI vaccines, as co-administration with other EPI vaccines showed a much lower induction of IFN-γ (Ota et al., 2011) while polio vaccine has been shown to reduce responses to BCG (Ota et al., 2002; Sartono et al., 2010). On the other hand, BCG given at birth has been shown to boost responses to other EPI vaccines, especially to hepatitis B in The Gambia (Ota et al., 2002), whilst it did not boost hepatitis B antibody levels in Australian infants (Ritz et al., 2013). These data indicate the importance of understanding vaccine-vaccine interactions to determine the optimal vaccine schedule for each country (as differences in host genetics plays a role in immune responses). Women tend to develop stronger immunity when compared to males as they have higher antibody levels and stronger cell-mediated immunity (Moxley et al., 2002), whether the same applies to infants is less clear. Geometric mean titres for measles antibodies after measles vaccination in adolescents showed a 50% higher titre in females when compared to males (Green et al., 1994). Indeed a 2-fold increase in female but not male mortality led to withdrawal of the high titre measles vaccine (HTMV) by WHO (Aaby et al., 2003). Contrary to the HTMV study, studies in West African infants have in general shown beneficial effects on females after vaccinations with live vaccines including measles vaccine (Roth et al., 2006; Aaby et al., 2011), and a detrimental effect after killed vaccines (Aaby et al., 2003). These effects are

also much more prominent depending on the vaccine that was last received (Hirve et al., 2012), thus making sequence of immunization very important.

6.1.2 Effect of BCG vaccination on the tuberculin skin test (TST)

The tuberculin skin test (TST/Mantoux test) is a standard test for measuring infectivity and /or exposure to *M.tuberculosis (Mtb)* by injecting purified protein derivative (PPD) intradermally and measuring induration (in mm) 48-72 hours after injection which is caused by a delayed-type hypersensitivity reaction to the antigen. However, TST reactivity can also indicate BCG vaccination and exposure to non-tuberculous mycobacteria (NTM), since induration (reaction) provides a measure of overall antimycobacterial immunity (Cobat et al., 2012). The "cut-off" to determine positivity as an indicator of infection with Mtb is not well defined and some studies have suggested that BCG vaccination can lead to a TST induration >10mm, with Chan et al. suggesting an induration of 21mm to be a more appropriate cut-off for the first year of life in Taiwanese infants (Santiago et al., 2003; Chan et al., 2008). It has been shown that BCG vaccinated infants with a positive TST induration and a BCG scar had lower all cause mortality rates suggesting a correlation with better immunity to TB (Roth et al., 2006). However, cross-reactivity with non-tuberculous mycobacteria found in the environment can affect TST reactivity. For example, Ota and colleagues found 22% of BCG naive infants (4.5 months old) and 20% of BCG-naïve adults had TST induration >10mm (Ota et al., 2006, 2007). However another study in a peri-urban setting found no TST reactivity in naïve infants at the same age (Burl et al., 2010).

Thus in this chapter we discuss the effect of BCG vaccination at 6 weeks of age on EPI vaccine antibody responses, including sex differences after vaccination. The effect of delayed BCG vaccination till 6 weeks on TST induration is also described.

Hypothesis:

- BCG vaccinated infants at 6 weeks would have higher antibody levels to other EPI vaccines when compared to BCG naïve infants
- BCG vaccination at 6 weeks would lead to higher indurations when compared to BCG naïve infants

Aims:

- Measure EPI antibody levels in BCG vaccinated and unvaccinated infants
- Investigate effect of BCG vaccination at 6 weeks on TST induration when compared to BCG naïve infants.

6.2 Methods:

6.2.1: EPI antibody levels

6.2.1.1 Polio neutralizing antibody levels

In order to examine the effect of BCG on polio vaccine immunity, we measured neutralizing antibody titres to polio 1 and 3 (the serotypes present in the vaccine given to Gambian infants) by culturing Human Epithelial-2 (Hep2) Cincinnati cells and measuring the cytopathic effect of the virus on these cells in the presence of human plasma. Antibody titres were measured after five days of incubation at 37°C, 5% CO_2. Fig 6-1 shows the cytopathic effect of the virus on the cells. The lowest possible measurable antibody titre was 8 and the protective cut-off value was a titre of >8; an antibody titre less than 8 (positive at 8, meaning the titre is less than 8) was given as 8. The highest possible titre was 1024 and any sample still negative at 1024 was given 1024 as the titre.

6.2.1.2 Hepatitis B surface antibody and DTP antibody levels

We measured surface antibody levels to hepatitis B (HBsAb) using a commercial kit (Diasorin, Italy) as described in Chapter 2 Section 2.4.2. 10 IU/mL is the protective level for hepatitis B whilst the highest measurable level was 1000 IU/mL. For analysis purposes all subjects with levels below 10 IU/mL were adjusted to 10 IU/mL and those with >1000 IU/mL were adjusted to 1000 IU/mL.

We measured antibody levels to DTP vaccine using an in-house multiplex immuno assay (MIA) technique.

6.2.2 Tuberculin Skin Test (TST)

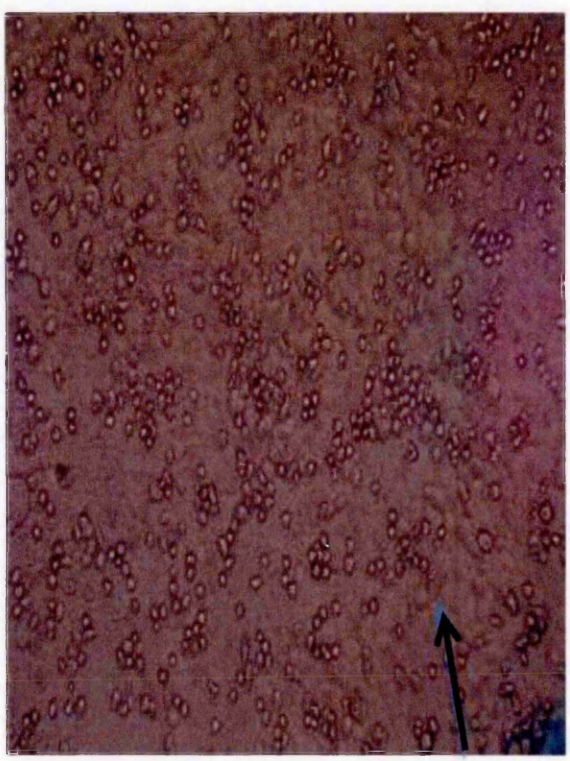
TST was done on all infants at 18 weeks, 2 weeks after the last dose of pentavalent vaccine was given. Induration was read longitudinally and transversely and the average induration (mm) recorded. Due to recent BCG vaccination an induration >10mm was considered positive and suggestive of Mtb infection.

6.3 Results

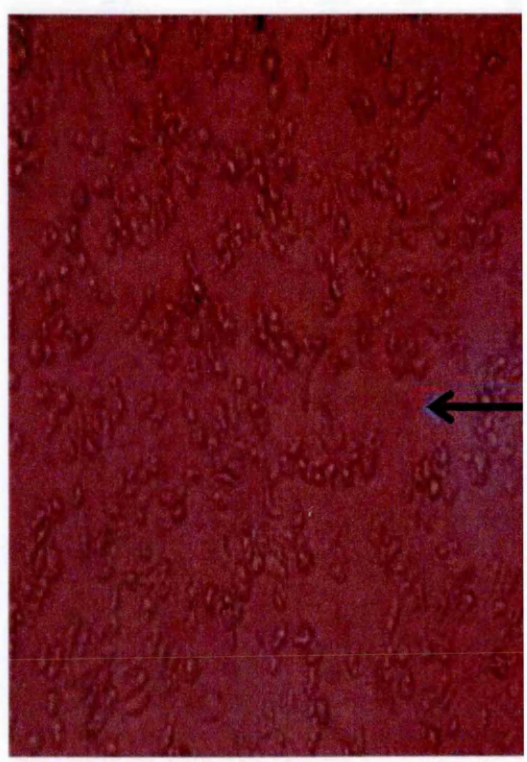
6.3.1 Vaccine antibody levels

6.3.1.1 Polio neutralizing antibody assay

We analyzed 61 unvaccinated and 62 vaccinated infants at baseline (pre-BCG vaccination), 59 unvaccinated and 62 vaccinated at 1-week post-vaccination, and 53 unvaccinated and 52 vaccinated at 12-weeks post-vaccination.



No Cytopathic effect (visible monolayer)- Arrow



Cytopathic effect (no visible monolayer)- Arrow

Figure 6-1: Cytopathic effect of Polio virus on Hep2 Cincinnati cells in the presence of plasma, visible monolayer indicates no cytopathic effect (negativity) whilst no monolayer indicates a cytopathic effect (positivity). Arrow indicates presence or absence of a visible monolayer.

By baseline, all infants had received one dose of OPV and four doses by 12 post-vaccination. At baseline, 66% of the vaccinated group and 62% of the unvaccinated group were protected for Polio 1 while only 35% of the vaccinated and 25% of the unvaccinated were protected for Polio 3 (no significant differences). These levels were significantly increased in both groups by 12 weeks post-vaccination with 94% of the vaccinated and 98% of the unvaccinated protected for Polio 1 and 97% and 85% for Polio 3 for the vaccinated and unvaccinated groups respectively. No differences were observed in antibody levels between the two groups over time, however differences were observed within both groups from 1 week post-vaccination to 12 weeks post-vaccination, when protective levels increased significantly (Fig. 6-2). We analyzed for sex differences in the antibody titre levels but did not find any differences between the sexes in the two groups at any time point (data not shown).

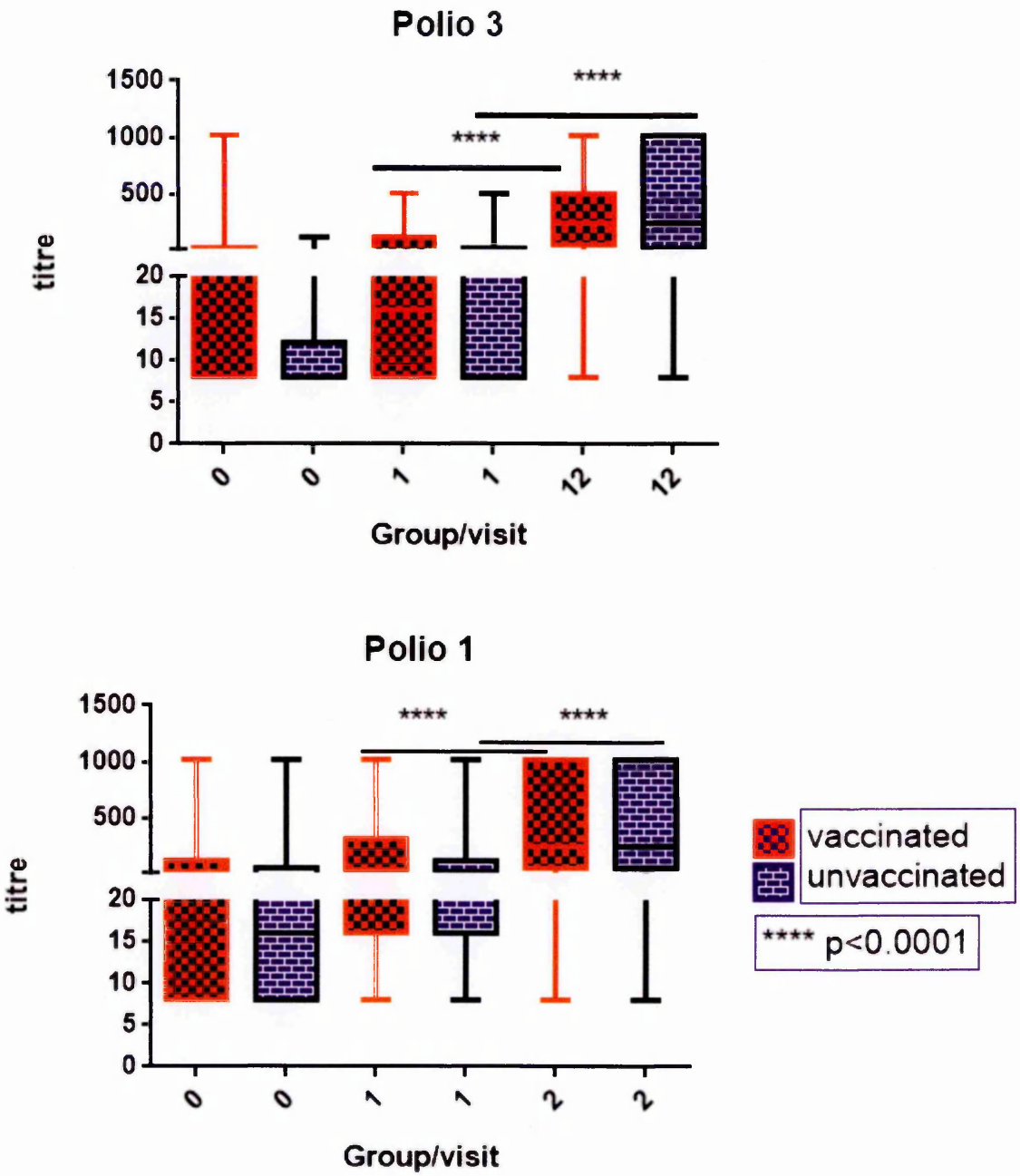


Figure 6-2: Antibody titres of Neutralizing Polio antibodies in the vaccinated and unvaccinated groups, at 0 (baseline/6 weeks), 1 (1 week post-vaccination/7 weeks) and 12 (12 weeks post-vaccination/18 weeks).No differences were observed between the groups over time. Data is representative of all study subjects.

6.3.1.2 Hepatitis B surface antibody levels in BCG vaccinated and BCG naive infants

At baseline, all infants had received a dose of HepB vaccine at birth, and by 12 weeks post-vaccination they had received an additional 3 doses at 2, 3 and 4 months. We observed similar antibody levels at baseline for both the vaccinated and unvaccinated group with only 17% of vaccinated subjects and 21% of unvaccinated having protective levels. There was little difference by 1 week post-vaccination but by 12 weeks post-vaccination, after 4 doses of the vaccine, all subjects had protective antibody levels against Hepatitis B (Median (IQR): BCG vaccinated: 1 week post-vaccination -10 IU/mL (10-717.8), 12 weeks post-vaccination -578.0 IU/mL (10.3-1000) $p<0.0001$; unvaccinated: 1 week post-vaccination -10IU/mL (10-698.5), 12 weeks post-vaccination -493.2 IU/mL (43.8-1000) $p<0.0001$ (Fig 6-3)).

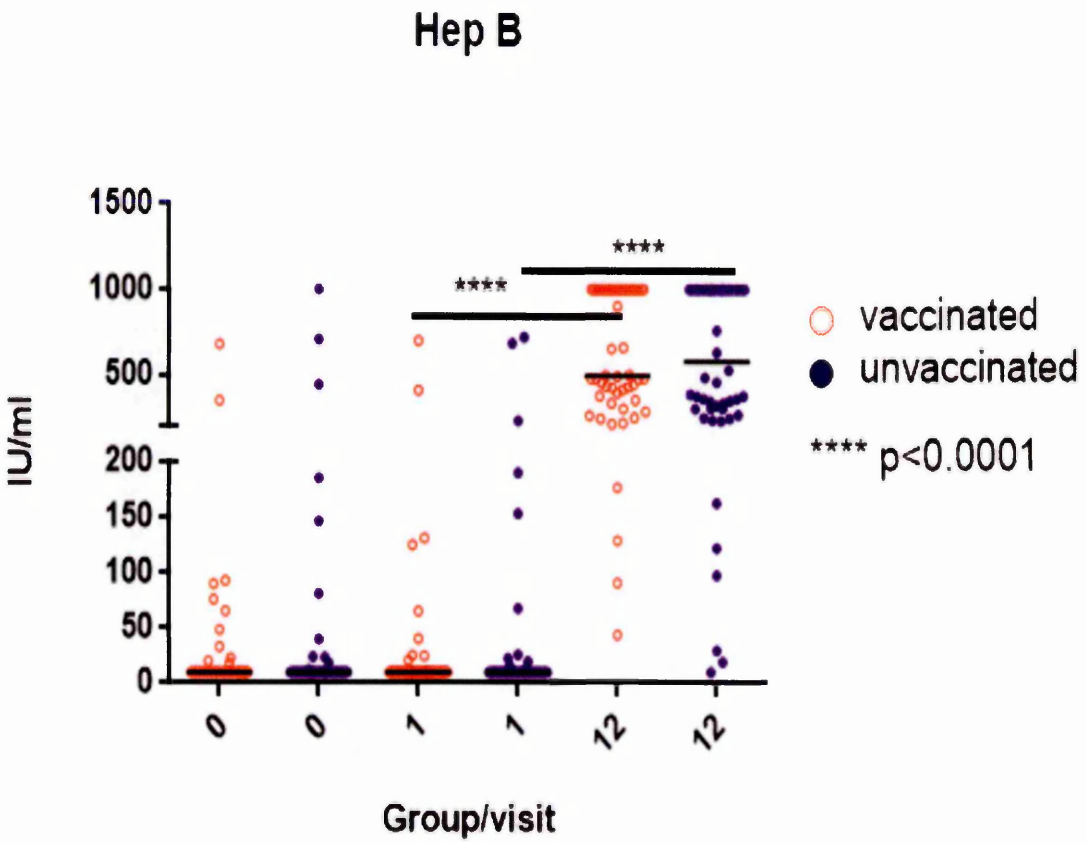


Figure 6-3: Hepatitis B surface antibody levels in BCG vaccinated and unvaccinated infants at 0= baseline/ 6 weeks, 1=1 week post-vaccination/7 weeks and 12=12 weeks post-vaccination/ 18 weeks. Line indicates median.

Interestingly, unvaccinated males at 12 weeks post-vaccination had considerably higher antibody levels than the unvaccinated females (median (IQR): males-1000 IU/mL (10.3-1000); females-352.9 IU/mL (19.2-1000), p=0.02; Fig. 6-4).

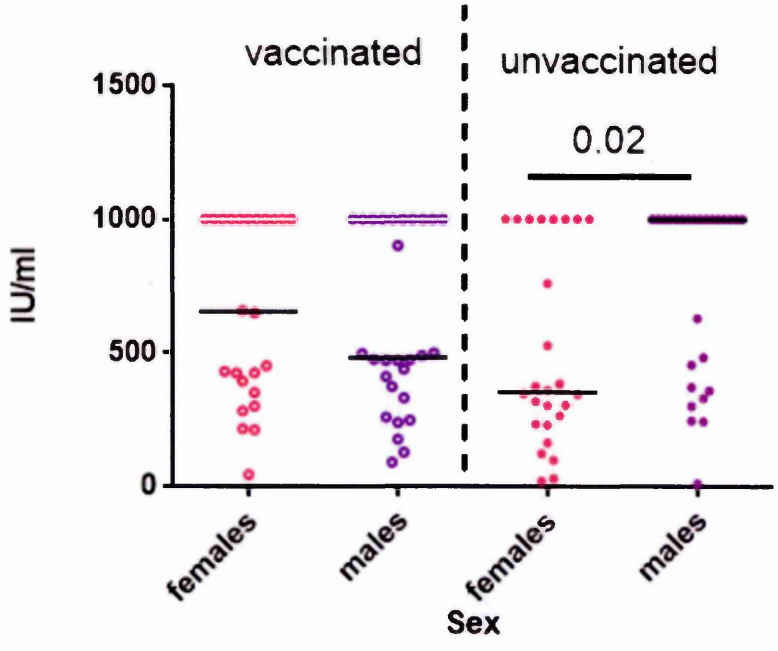


Figure 6-4: Gender differences from Hepatitis B surface antibody levels in BCG vaccinated and unvaccinated infants at 12 weeks post-vaccination/ 18 weeks. Line indicates median. Data anal yzed using Mann Whitney U test and representative of all study subjects

6.3.1.3 Antibody levels to Diphtheria, Tetanus and Pertussis

The cut off for protection for pertussis toxin (Ptx) antibodies is 25 EU/mL: at baseline 6% of all subjects had protective levels but by 12 weeks post-vaccination , 94% of vaccinated but only 84% of the unvaccinated had protective levels ($p<0.0001$ for both groups). The protective cut-off value for diphtheria toxoid (Dtx) antibodies was 0.01 IU/mL, and 44% of the vaccinated and 45% of the unvaccinated were protected at baseline and by 12 weeks post-vaccination 100% of study subjects had protective levels (Fig 6-5b). The protective cut-off level for tetanus toxoid (Ttx) antibody levels was 0.01 IU/mL and 100% of the vaccinated infants were protected and only 1 unvaccinated infant (0.016%) was unprotected at baseline; while all the infants had protective levels by the third visit, with no differences between the groups despite increase in antibody levels by 12 weeks post-vaccination ($p<0.0001$) (Fig 6-5c). We did not find any sex differences for any of the DTP antibody levels at any time-point within or between groups.

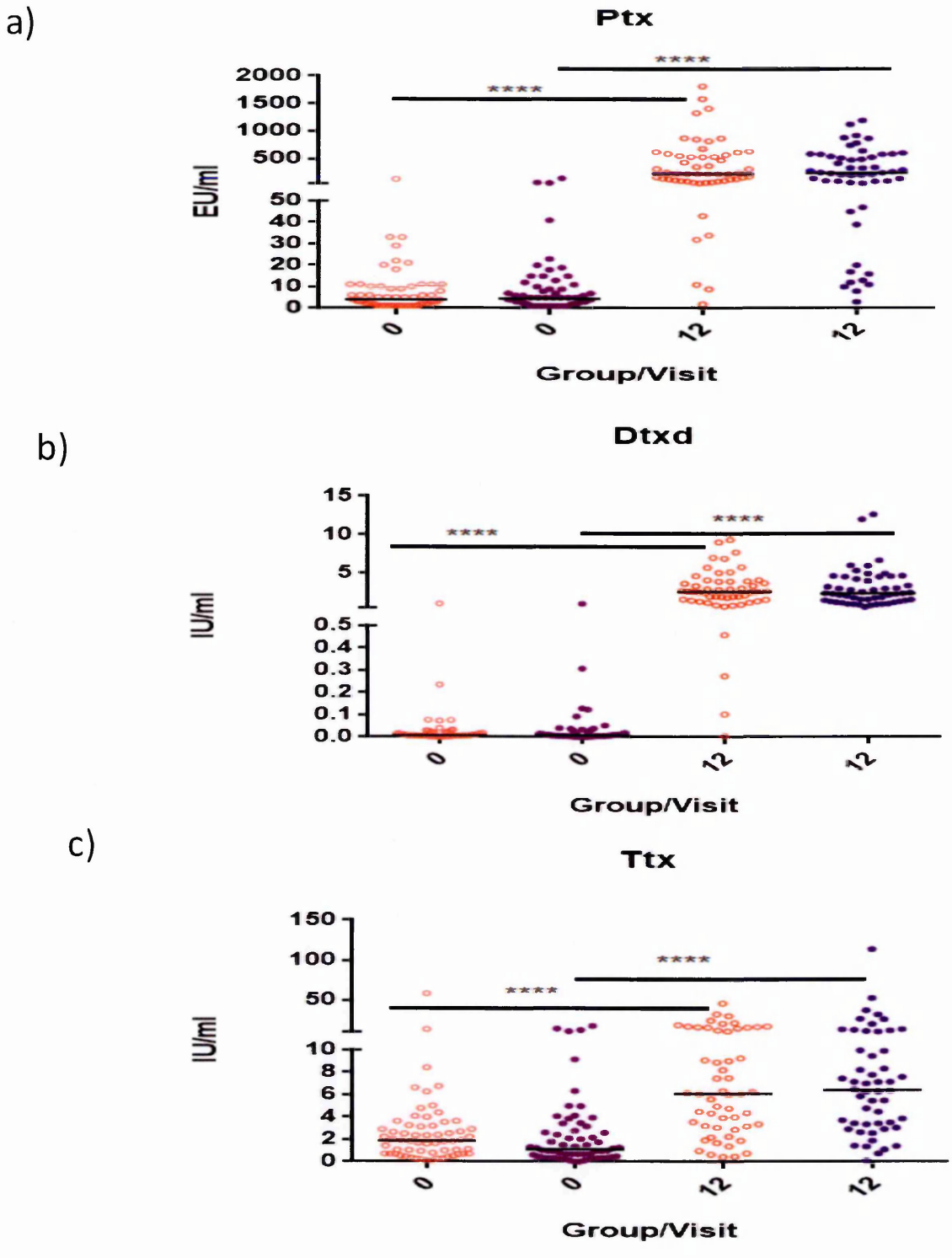


Figure 6-5: Antibody levels to diphtheria, tetanus and pertusis by MIA. No effect of BCG was observed on levels to these antigens. Open circles represent the vaccinated, closed circles represent unvaccinated infants. 0= baseline/6 weeks, 1= 1 week post vaccination/ 7 weeks, 12= 12 weeks post vaccination/18 weeks. ****= $p<0.0001$

6.3.2 Effect of BCG vaccination on the Tuberculin Skin Test

We measured TST induration in all subjects at 18 weeks in order to assess the effect of delayed BCG vaccine on tuberculin reactivity. This time-point was 12 weeks post BCG vaccination in the BCG-vaccinated group and just prior to the unvaccinated group receiving their BCG dose. 45 (87%) unvaccinated subjects were non-responders (i.e. had an induration of 0mm), 5 (9.6%) had an induration between 0 and 10 mm and 2 (3.8%) had an induration of ≥10mm (and thus classified as TST positive). In the BCG-vaccinated group, significantly more subjects were TST+ as expected, 16(30%) having an induration ≥10mm ($p<0.0001$; Fig 6-6a), despite this, 21(40%) of the vaccinated infants were anergic (induration=0mm) and 16 (30%) had an induration between 0-10mm. Infants with indurations ≥10mm were investigated for TB exposure but no cases were found. Interestingly, in the vaccinated group, males had higher induration compared to females (median (IQR): males=8.5mm (0mm-16mm); females=1.3mm (0mm-13mm), $p=0.012$) but no difference was observed in the unvaccinated group between the sexes (median (IQR): males= 0mm (0mm-9.5mm); females=0mm (0mm-12mm). The vaccinated males and females had higher induration than the unvaccinated males and females after stratification based on sex (males- $p<0.0001$), females-$p=0.02$) (Fig6- 6b).

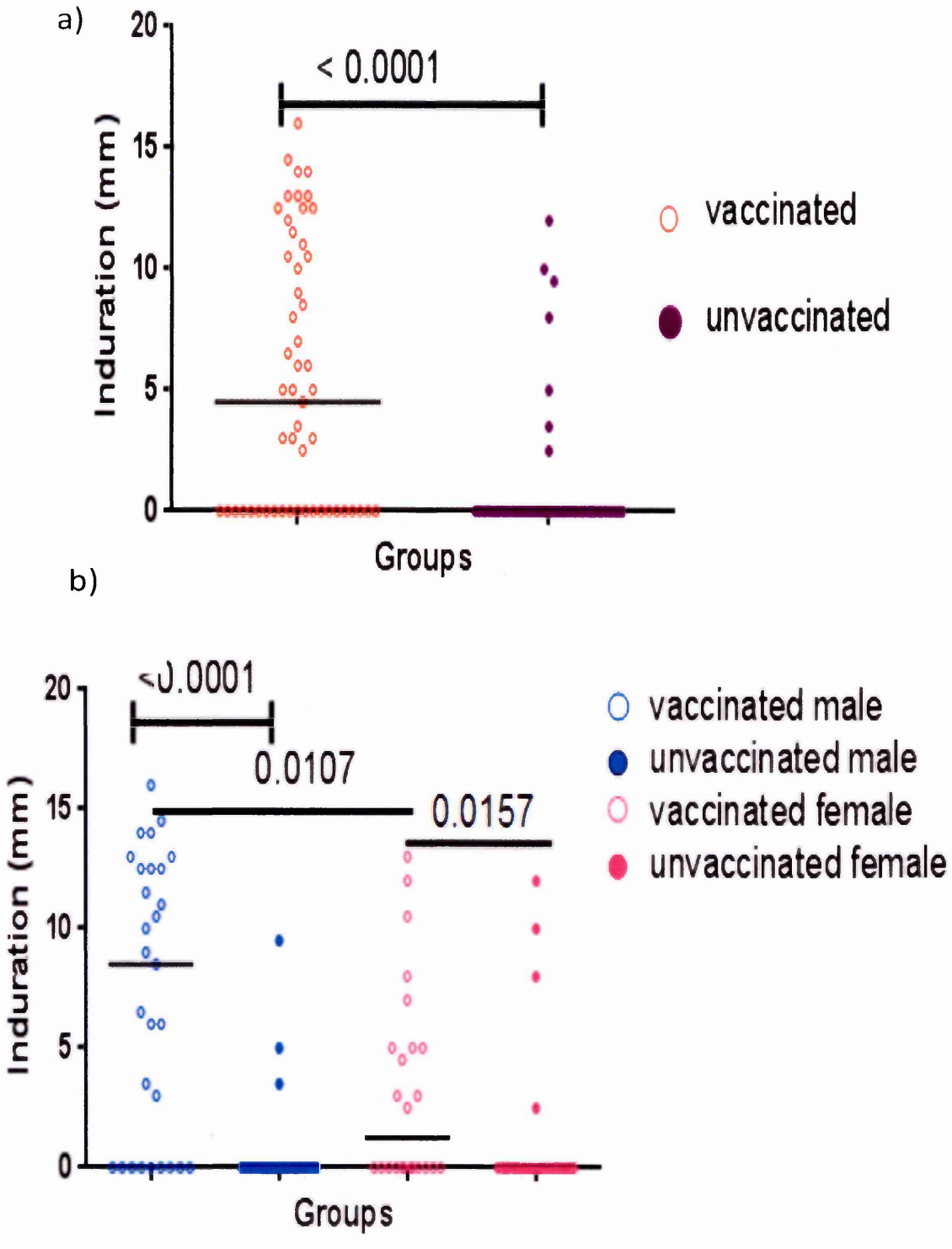


Figure 6-6: a) Overall differences in TST induration between the vaccinated and unvaccinated groups; at 18 weeks of age, the vaccinated infants had higher induration than the unvaccinated, b) Sex differences in TST, males and females of the vaccinated group had higher induration than their unvaccinated counterparts and males in the vaccinated group had higher induration than the females.

6.4 Discussion

We measured antibody levels to different EPI vaccines and related this to BCG vaccination status. We expected that the vaccinated group would have higher EPI antibody levels when compared to the BCG unvaccinated group but we found no difference between the two groups. Previously, BCG has been shown to boost antibody levels to HepB and OPV vaccines but not to tetanus and diphtheria vaccines in Gambian children (Ota et al., 2002); whilst in Australian children, it did not boost levels to HepB, tetanus and Hib vaccines, but boosted levels to some pneumococcal vaccine serotypes (Ritz et al., 2013). Whilst we used the same method of analysis for polio and HepB as Ota et al, we did not find the differences they observed in regards to polio 1 virus or hepatitis B antibodies. This may be due to differences in BCG vaccine strain administered but is more likely associated with the different vaccination schedule we used. First of all, the infants who were vaccinated were 6 weeks older than in previous Gambian studies. This means the infants' immune system would be different with an increase in adaptive immunity present, and also the timing of the vaccines would affect responses to the other EPI vaccines. For instance, the previous studies gave BCG together with the other EPI vaccines, whilst we gave BCG after the first dose of Polio and HepB but 2 weeks before the other EPI vaccines (DTP). Differences in vaccine strains makes direct comparisons between our study and previous studies difficult as the various strains perform distinctly from each other when used in diverse places and have different properties such as virulence factors, immunogenicity and efficacy (Fine, 1999; Behr, 2002; Corbel et al., 2004; Davids et al., 2006; Leung et al., 2008; Ritz et al., 2009; Anderson et al., 2012). Importantly however, protective antibody levels to Polio, TT and HepB were achieved in all subjects regardless of vaccine group, showing that the delay in BCG vaccine is not detrimental to responses to other vaccines. Whilst non-specific boosting effects are a 'bonus' of the BCG vaccination, their absence is not important in terms of antibody responses if protective levels are reached anyway.

Despite arguments that the presence of maternal antibodies might be partially responsible for sub-optimal vaccine efficacy in infants (Siegrist et al., 1998), they play a vital role in protection against invasive diseases in infants (Saffar et al., 2008). Expectant Gambian mothers receive a tetanus vaccine booster upon their first visit to the antenatal clinic and a second dose is recommended 6 months later, which should fall during the third trimester. However, many pregnant mothers do not visit antenatal clinics early thus most only receive one dose of tetanus during the second or third trimester (Dr. Jane Adetifa, personal communication). At 6 weeks of age, before receiving the first dose of the DTwP vaccine, all but one infant in our study had protective Ab titres against tetanus; and by 18 weeks of age, after receiving all three doses of the DTwP vaccine, all the infants had protective anti-tetanus titres. This indicates the importance of protection conferred by maternal antibodies in our setting. And the delay in BCG vaccination did not affect this. It is a limitation in TB studies that antibodies are not protective and therefore analysis of Mtb-specific antibodies (such as the lipoarabinomannan (LAM) urine test) cannot be used in the same way as the HepB or polio assays. In addition, our study did not assess the long-term effects of BCG vaccination so we cannot ascertain the clinical end-points for any diseases within our study group. Unvaccinated males had significantly higher HepB antibody levels when compared to the females in the same group, contrary to current literature which suggests females tend to have higher antibody levels than males (Green et al., 1994; Moxley et al., 2002). These latter studies were conducted in adults and the situation may be different in infants.

Both BCG vaccinated and unvaccinated subjects had TST induration indicative of vaccination and or exposure to TB or non-tuberculous mycobacteria, but induration was significantly higher in the vaccinated than the unvaccinated showing the effect of BCG vaccination on the TST readout, an indication that the vaccine used in this study did work. The cut-off used to suggest TB infection was 10mm; all subjects with >10mm induration were followed up and investigated for TB exposure and none of them had a history of a close contact with anyone with signs and symptoms of TB. Indeed a previous study in

BCG naive Gambian infants found that 22% of infants were TST positive at 4.5 months (Ota et al., 2006); in contrast another Gambian study which found no reactivity in unvaccinated infants despite the presence of non-responders (anergic) in infants vaccinated at birth (Burl et al., 2010). Chan et al suggested a cut-off of 21mm as a strong predictor of Mtb infection in BCG vaccinated infants up to 1 year of age; and 18mm was proposed as a cut-off from 12-20 months of age; while suggesting that any induration below this could be attributed to vaccination (Chan et al., 2008). According to this guideline we did not observe any possible cases of Mtb infection using TST as a diagnostic tool. In addition, all infants in our study with a TST >10mm were followed up and investigated for TB exposure; none of the infants had a history of a close contact with anyone with signs and symptoms of TB, and the infants themselves did not have any signs of the disease. Therefore the responses we observed were probably due to either BCG vaccination in the vaccinated subjects or exposure to environmental mycobacteria in the unvaccinated.

In summary, we found that there was no effect of BCG vaccination at 6 weeks of age on other EPI vaccines given from 2 weeks after BCG; but did find that unvaccinated males had higher antibody responses to HepB vaccine than unvaccinated females, and vaccinated males had higher TST induration than their female counterparts.

Chapter 7: General discussion

BCG is currently the only licensed vaccine against tuberculosis and is administered to over 90% of children in TB endemic regions; although efficacy rates are not consistent between or within countries. This lack of consistent and reliable protection has galvanized the search for new vaccination strategies using recombinant technology or heterologous vector based prime-boost regimens (Ottenhoff & Kaufmann, 2012; Kaufmann, 2013). However, in order to develop optimal vaccination strategies it is important to determine the precise role of innate and adaptive immunity in protection to BCG. Therefore, the aim of this study was to analyze responses to both vaccine-specific and unrelated antigens in infants who received BCG at 6 weeks of age compared to those who had delayed vaccination to 18 weeks.

Despite differences in Th1 responses found in previous studies between BCG vaccinated cohorts and unvaccinated cohorts (Marchant et al., 1999; Vekemans et al., 2001; Soares et al., 2008; Burl et al., 2010), we found few differences between our BCG vaccinated and naïve groups. This could be attributable to three main factors. Firstly, pre-sensitization to environmental mycobacteria blocking/masking immune responses following BCG vaccination is a prominent hypothesis (masking hypothesis) (Brandt et al., 2002; Fine et al., 1995) and it has been observed that exposure might lead to an attenuated effect on the immune response after BCG vaccination (Black et al., 2002; Ota et al., 2002; Burl et al., 2010), particularly in TB-endemic settings. All babies in this study had BCG vaccination delayed, with the vaccinated group only receiving the vaccine at 6 weeks of age compared to other studies where infants received the vaccine at birth (as per normal EPI schedule). Secondly, cytokine levels were measured after overnight cultures and this might not be enough time to see differential effects, particularly in regards to the adaptive response. However, we did see higher Th1/Th2 cytokine ratios in the vaccinated group when compared to the unvaccinated infants and the unvaccinated group had increasing Th2 cytokine levels over time suggesting a bias towards Th2 levels in the BCG-delayed

group. Thirdly we measured responses to vaccines one week post vaccination and a previous South African study showed that BCG vaccine responses peak at 10 weeks post-vaccination and there is very little responses at baseline (3 week post-vaccination)(Soares et al., 2013), hence the time we bled after vaccination might be too soon to see responses. Surprisingly, the unvaccinated infants had higher Th1 and Th17 but no difference in innate cytokine levels compared to the vaccinated infants following TLR agonist stimulation and few changes were observed at the different time-points. These results are also in contrast to a previous cross-sectional study in Gambian infants (Burl et al., 2011), which showed higher pro-inflammatory responses at birth, decreasing over time.

In South Africa, a better T cell response was observed in 1 year olds vaccinated at 10 weeks compared to those vaccinated at birth (Kagina et al., 2009). In contrast, a study from Uganda supports our findings showing reduction in T cell responses in infants who delayed vaccinated to 6 weeks in Uganda (Lutwama et al., 2013). One of the major limitations of the latter study is the socioeconomic background of the study subjects: those vaccinated at birth were born in hospitals and those vaccinated at 6 weeks were born at home. All our study subjects came from the same socioeconomic background, so this was unlikely to be a contributing factor in the low response to PPD in the BCG vaccinated infants. However, it does suggest that masking with environmental bacteria, differences in TB exposure and differences in host genetics all contribute to the differences seen between study sites.

Human studies looking at vaccine efficacy have used polyfunctionality as a correlate of protection for almost a decade now. Previous studies in murine models have shown polyfunctionality to be correlated with protection against TB despite it being dose dependent (Forbes et al., 2008;Aagaard et al., 2009). Interestingly, we did not find any differences in our study in terms of polyfunctionality.

Whilst we could not determine a disease end-point in our study, we did assess TST indurations at 18 weeks to determine possible Mtb infection in both groups. High TST indurations were seen in the unvaccinated infants suggesting mycobacterial exposure and priming. However, a previous Gambian study observed that 22% of infants had TST induration despite being BCG naïve and none were infected with Mtb suggesting an impact of non-tuberculous mycobacteria on the TST reading. Thus, we used a cut-off of 10mm or above as an indicator of infection with TB and all infants with a TST >10mm were investigated for TB exposure. Importantly, none of the infants in either group were exposed to TB or showed signs/symptoms of infection. Indeed, a recent study in the same study setting found low exposure of 18 month old-infants to TB (Odutola et al., 2012)..

One of the major limitations in the present study was the lack of a birth cohort whereas all the previously stated studies had a birth cohort to compare with the delayed group. Another difference with the previous studies, especially The Gambian studies is that other EPI vaccines were given at the same time, i.e. at birth, at 2 months or at 4 months, whilst in this study BCG was given alone at 6 weeks, 6 weeks after the first doses of the EPI vaccines and 2 weeks before the second dose. The lack of a boosting effect on the other antibody levels could be attributed to this since effects seen previously might have been due to the interactions of all the different vaccines given together and not necessarily the BCG vaccine. Another limitation is that we did not perform a functional assay to assess responses in the BCG-vaccinated infants. For example, the BCG Lux assay measures killing of mycobacteria in culture after vaccination and has been shown to work in TB endemic settings (Kampmann et al., 2000; Newton, Martineau, & Kampmann, 2011; Burl et al., 2013). It would have been interesting to observe differences in mycobacterial killing between the two groups and the effect delaying BCG till 6 weeks might have on this despite the low cytokine levels observed. Finally, we did not assess responses in the infants who had BCG vaccination delayed until 18 weeks of age. This would have been

important to determine the influence of even further delay on BCG responses and responses to other EPI vaccines and may have helped to address the masking hypothesis.

In conclusion, BCG Russia given at 6 weeks of age showed no enhancement of Th1 responses but decreased IL-10 responses after TLR agonist and unrelated pathogen stimulation compared to BCG unvaccinated infants. These effects were more prominent in females when compared to their male counterparts. In contrast to previous studies on BCG vaccination at birth, there was no effect on other EPI vaccine antibody levels when given at 6 weeks with all infants reaching protective levels by 18 weeks of age.

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